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UNIVERSITY OF ALBERTA

DIETARY CONTROL OF INTESTINAL EXPRESSION OF GLUT2 AND GLUT5 IN RAT

BY

LINA CHO-LEAM ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

DEPARTMENT OF PHYSIOLOGY

Edmonton, Alberta

Spring, 1994

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **DIETARY CONTROL OF INTESTINAL EXPRESSION OF GLUT2 AND GLUT5 IN RAT** submitted by **LINA CHO-LEAM** in partial fulfillment of the requirements for the degree **MASTER OF SCIENCE.**

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April 22, 1994

Dedicated to the memory of my grandparents,
Sieh-Yueh Wen-Cho and Lien-Yi Cho,
for their kindred spirits.

ABSTRACT

The small intestine can adapt to altered dietary carbohydrate content by changing its glucose transport capacity and this response is effected by inducing or repressing the expression of hexose transporter proteins. However, the underlying molecular mechanism controlling this carrier expression has not been elucidated. Glucose entry into enterocytes is mediated by the Na^+/glucose cotransporter SGLT-1, a protein which utilizes the sodium electrochemical gradient to move glucose into the cell against a concentration gradient. Efflux of glucose and fructose out of the enterocytes into the portal circulation is then mediated by one or more facilitated carriers which are members of a supergene family referred to as 'GLUT'. To date, both GLUT2 and GLUT5 mRNA have been detected in significant amounts within the small intestine. GLUT2 has been localized to the basolateral membrane (BLM) of the enterocyte but GLUT5, a fructose transporter, is probably present in the brush-border membrane (BBM).

In this work, Northern hybridization analysis was employed to measure the abundance of GLUT2 and GLUT5 mRNA in the duodenum, jejunum, ileum and colon of the rat. By using RNA antisense probes, a 2.8 kb GLUT2 mRNA transcript was detected predominantly in the jejunum, with significantly less in the duodenum and ileum and none in the colon. GLUT5 northern blots showed two mature transcripts, sized at 2.0 kb and 2.8 kb which were differentially expressed along the horizontal axis of the small intestine. This could reflect tissue specificity and/or functional changes of the GLUT5 carrier required to meet the demands of that particular region.

When rats were switched from low (31%) to high (59%) carbohydrate diet (LC,HC), jejunal GLUT2 mRNA levels increased ~2 fold after 24 h and then declined to the level of the LC diet fed animals. In parallel, GLUT2 carrier protein was measured by western blotting and showed progressive upregulation in the jejunum over the week of HC diet consumption. This strongly suggests that gene transcription is not the primary mechanism sustaining the increased BLM transport

capacity observed in dietary adaptation, but rather, involve changes in GLUT2 expression mechanisms operating at the post-transcriptional level.

Immunocytochemical detection localized GLUT5 to the brush-border membrane of intestinal enterocytes. This integral membrane protein exhibited a gradient of expression along both the crypt-villus axis and horizontal axis of the small intestine from duodenum to ileum. The expression predominates in the upper third of the villus and the proximal regions (duodenum and jejunum) of the small intestine. This is possibly related to the gradient of the lumenal fructose concentration which would prevail during a meal.

GLUT5 jejunal mRNA levels responded to the switching from LC to the HC diet in a parallel fashion to that seen for GLUT2. There was a small (~30%) transient increase after 24 h of HC feeding, but thereafter levels were maintained relatively steady.

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I.1 Introduction

The mammalian intestinal epithelium exhibits a remarkable capacity to adapt to its changing environment. Unlike most other somatic cells, the gut can be exposed to a wide variation in substrate composition and concentrations, depending upon the diet. This requires operative mechanisms which can accommodate changes in dietary composition for both short (duration of a meal) and on a longer term basis (changes in diet sustained over days to weeks). This contrasts with the majority of vertebrate tissue, for which the cells are exposed to an internal medium whose composition is held relatively constant. Because whose substrate levels are maintained within a narrow range, there is no need for substantial substrate-dependent regulation or it is designed to maintain intracellular homeostasis.

Glucose absorption by the intestine epithelial cells is a two step process. The brush-border Na+/glucose symporter transports glucose from the lumen into the cell against its concentration gradient and the import is driven by movement of sodium ions down their electrochemical gradient. Release of glucose across the basolateral membrane and into the blood is catalyzed by GLUT2, a facilitated diffusion glucose transporter.

The concept of dietary adaptation describes the up- and down-regulation of nutrient transport in response to changes in concentration of dietary substrates. This phenomenon has been demonstrated for glucose transport across the intestinal brush-border and basolateral membrane of both the mouse and rat. For instance, Diamond and his colleagues (28) showed that raising the carbohydrate concentration in the diet increased sodium-dependent glucose transport 2 fold. This increase was paralleled by a similar change in glucose transporter site density as determined by sodium-dependent glucose inhibitable binding of phlorizin to the intestinal mucosa (37). Cheeseman and Harley (19) also showed a similar response to increased dietary carbohydrate for glucose transport across the BLM. Again cytochalasin-B binding to the BLM indicated an increased expression of carriers at this pole of the cell. Both

studies independently concluded that the modulation in glucose transport is a consequence of an increase in Vmax, the transport capacity, with no apparent change in Km, the Michaelis-Menten affinity constant. Therefore, it has been proposed that the cellular mechanisms by which absorptive capacity is up-regulated occur primarily through changes in the number of transporter copies in the plasma membrane. Such changes could be mediated through a number of processes including: a change in rate of carrier biosynthesis, degradation, or membrane insertion (17,55).

Hence, the primary focus of this project was to investigate the underlying molecular mechanism controlling glucose transport in response to alterations in the diet. There are many mechanisms by which control of gene expression can be regulated along the pathway of RNA synthesis to insertion of the final protein product and they include transcription, pre- and post-transcriptional controls. I chose to focus on just two, gene transcription and possible post-transcriptional events. To achieve this, complementary RNA probes were used with Northern blot analysis to search for changes in steady-state mRNA levels in response to altered dietary carbohydrate. The carrier protein expression was then compared with the mRNA levels. The data indicate that the increase in the number of carriers is the result primarily of changes which occur post-transcriptionally i.e after the mRNA for the transporter protein has been synthesised within the enterocyte.

This literature review encompasses three main areas, 1) modes of adaptive response of intestinal glucose transport in various situations, and physiological states, 2) an understanding of *macro-* and *micro-* mechanisms which influence expression of glucose transport and other intestinal proteins, 3) a summary of the molecular biology of facilitative glucose transporters with emphasis upon GLUT2 and GLUT5 glucose carriers.

I.2 Dietary Adaptation of Intestinal Glucose Transporters\ Reversible and Specific Controls

Intestinal glucose transport is reversibly and specifically regulated by dietary levels of its substrate. It has been established that monosaccharide absorption in

rodents is stimulated, *in vivo* and *in vitro* (8,41,47) in response to increased dietary carbohydrate content. To date, this substrate-dependent induction has been characterized separately for both poles of the enterocyte, the brush-border membrane (BBM) and the basolateral membrane (BLM) in the mouse and rat respectively (19,37). By using an everted intestinal sleeve technique, Karosov and Diamond (61) clearly proved that the effect of high dietary carbohydrate was specific in altering active D-glucose transport in the BBM and was not due to non-specific changes mediated by a gross increase in mucosal mass or a change in passive glucose permeability. The kinetic basis of such processes has been attributed to the increase in the Michaelis-Menten constant, Vmax, without any alteration in the affinity constant, Km (29). Initial studies by the same group did demonstrate an increased Km, but this was later shown to be an artifact resulting from the influence of the unstirred water layer adjacent to the intestinal mucosa (28). The water layer acts as a physical barrier to the substrate being transported. This physical phenomenon results in experimental error in calculating the Km for the transported process because the fluid surrounding the mucosa reduces the concentration of substrate at the cell surface. The BBM active uptake of monosaccharide responded rapidly and reversibly to dietary changes in carbohydrate and depended on the luminal concentration of glucose. When the diet was switched from low carbohydrate (LC) to high carbohydrate content (HC), stimulation of transport was complete within 1 day. While switching back to LC, it took 3 days before down-regulation was complete (37).

Glucose transport across the basolateral membrane can also be modulated. Maenz and Cheeseman (71) simulated situations of acute hyperglycaemia to mimic diabetes and showed a significant increase in the capacity of the rat small intestine to absorb glucose. The induction is characterized by a 4.1 fold increase in transport after a 6 h vascular infusion of a high glucose solution, and a 1.8 fold increase in cytochalasin-B binding compared to that of non-infused rats (20); it was shown later by Miayamoto et al (73) that there was no change in GLUT2, a basolateral glucose carrier protein, levels. These results provide clear evidence that the exit of glucose

from the enterocyte can also be regulated. However, the effected response in short term hyperglycaemia is quite different from the response for rats given a high carbohydrate diet. With changes in the dietary carbohydrate levels, the capacity for glucose transport in the BLM was also altered, but the time course for upregulation was shown to be slower than that of the BBM in the mouse. At least 3 days of HC diet was required to produce a two-fold increase in the BLM hexose transport capacity. This up-regulation was maintained for the full 7 days of the dietary treatment (19). Kinetic analysis showed an increase in Vmax, with no change in the apparent affinity constant, Km, or passive permeability to glucose. Analysis of specific cytochalasin-B binding to these membranes show a parallel change in the number of glucose inhibitable binding sites, suggesting that adaptation to increased dietary carbohydrate is due to an increase in the number of glucose carriers in the BLM.

The cytochalasin-B inhibitable basolateral glucose transport system has also been characterized in cultured kidney LLC-PK1 epithelial cells and renal basolateral membranes in rabbit (23,76,78).

I.2.1 Reversible and Nonspecific Controls

The modulation of glucose transport in dietary adaptation is reversible and specific, however, conditions also exist whereby the transport of glucose is reversible but non-specifically upregulated. Gestation and lactation are two physiological conditions associated with increased energy requirements and hence increased food intake (62). Reports have shown that laboratory rat food intake increase 2-3 fold during these altered metabolic states which results in higher glucose and amino acid uptake in pregnant or lactating rats than controls (25). The mechanism of this adaptation to increased energy demand is primarily mediated by hypertrophy of the intestinal mucosa, which causes increases in the numbers of all nutrient transporters including those for glucose uptake. It was concluded by Fell and colleagues that these anatomic changes are due to the stimulation of cell division in the crypts leading to an increased overall villus height (36).

I.2.2 Irreversible and Specific Controls

The adaptive response of intestinal hexose transporters can also be irreversible. This results from changes in diet occurring at one critical period in an animal's development. There are two well known examples of intestinal sugar transport subjected to irreversible change, both occur during weaning (when the animal makes the transition from the mother's milk to its natural diet). For instance, fructose is absent from mammalian milk, and it first appears in the natural diet of herbivores and omnivores but not of carnivores at weaning. Buddington et al (11) showed that there is a parallel increase in fructose transporter activity at weaning in herbivores and omnivores but not carnivores. The second example is the decline of glucose transporter activity in carnivores but not in omnivores and herbivores, at weaning, which is attributed to the decrease of glucose content in the natural diet of the carnivore. Omnivores and herbivores maintain their ability to absorb glucose after their weaning off milk since glucose continues to be a potential major calorie-providing nutrient in their diets (11,60,85,100).

Previous studies have demonstrated a dramatic decline in the activity and abundance of intestinal Na+/glucose cotransporter (SGLT1) during the post-natal development of lambs. It appears that the decrease in glucose transport activity in the ruminant intestinal tract coincides with maturation of the rumen. In this organ, dietary carbohydrates are fermented into volatile fatty acids by the rumen microflora. Consequently, the amount of hexoses reaching the small intestine declines during weaning and are undetectable in the intestine of adult sheep (42).

I.3 Mechanisms of Dietary Adaptation

Studies of mechanisms involving dietary adaptation have previously focused on macroscopic changes to the intestine. These changes include the alterations in amount of mucosa associated with gross hyperplasia or hypoplasia of individual villi, and the alteration of total intestinal length (61,105). Dietary effect on parameters such as enterocyte migration rate (EMR) and life-span have also been examined, as these could affect transport activity non-specifically by contributing to the villus

height or the proportion of mature transporting cells (40). The overall consensus derived from these studies is that morphological changes do not necessarily parallel changes in transport capacity in dietary adaptation (95). On the microscopic level, the molecular basis of these changes is just beginning to be understood. Pilot studies in dietary adaptation have defined the parameter contributing to increased glucose transport, Vmax, and the glucose carrier site density has been measured along the horizontal axis (19,37) of the small intestine, as well as the crypt-villus axis (38,39), by using specific binding studies. In addition, the recent elucidation of sequences encoding the GLUT transporter family and SGLT-1 has provided genetic probes to examine the molecular events which regulate gene expression (5). This will allow further investigations to elucidate how molecular mechanisms facilitate changes to glucose transport capacity in response to external stimuli.

I.3.1 Macroscopic Mechanisms

The enterocytes lining the small intestinal mucosa have an average life-span of 68 h in the rat (14,47). These cells are therefore renewed every 2-3 days and are generated by continuous mitosis of a stem cell population located in the intestinal crypts. As enterocytes migrate toward the villus tip, they mature and acquire differentiated functions which include absorption of hexoses, amino acids, vitamins, and essential ions. It is believed that mature enterocytes eventually slough off into the intestinal lumen and are replaced by new ones (22). However, the precise process of the elimination of old epithelial cells at the villus tips remains unknown. Recent reports reveal a novel mechanism for disposing of effete epithelial cells in the small intestine of guinea pigs. It appears the old enterocytes are being phagocytosed by macrophages located in the lamina propria, leaving their apical cell portion in the epithelial lining. These cytoplasmic plates bridge between intact enterocytes and are subsequently pinched off into the lumen as the epithelial cells close to form new junctional complexes beneath them. Hence, the epithelial barrier in the villus tips is never destroyed during this renewal process (57).

The stem cells can differentiate into one of four epithelial cell phenotypes: 1)

absorptive enterocytes, 2) mucus-secreting goblet cells, 3) enteroendocrine cells and 4) Paneth cells (which migrate to the crypt base). Overall, the population of absorptive epithelial cells predominate, constituting ~90% of cells in the crypt compartment and >95% of cells on the villus (101). The intestinal mucosa is further characterized by a decreasing villus height from duodenum to ileum. This differential tissue gradient parallels regional decreases in cell migration time (time required for enterocyte to migrate from crypt to villus tip)(40).

The mechanistic basis of dietary carbohydrate adaptation is often assumed to be a specific effect of a substrate on carrier protein synthesis, membrane insertion or degradation. However, studies on non-specific effects of diet were investigated by Ferraris and colleagues to see whether villus height plays an integral part in the alteration of transport activity. The two factors contributing to villus height examined were: 1) cell migration rate (CMR), 2) life-span of enterocytes. Any reduction or increase of either parameter would ultimately result in changed transport activity. They concluded that both CMR and cell life-time are independent of diet when the protein content is maintained above the maintenance level (39).

It is not known whether the diet exerts a nonspecific effect by altering the biochemical properties and hence biophysical properties of the lipid membrane in which the carrier proteins are anchored (72). The influence of membrane lipids on protein mediated activities of biological membranes were first demonstrated in E. Coli and mycoplasma (81,87). By changing the biochemical and biophysical properties of brush-border membranes of the intestine, Brasitus et al. attempted to establish a relationship between activity of D-glucose transporters and membrane fluidity in diabetic rats (9,31,32). Unfortunately, it still unclear what role lipid dynamics play in modulating membrane transport processes in the intestine.

1.3.2 Microscopic Mechanisms

The well characterized increased rate of glucose transport in response to dietary composition was attributed to an increase in maximal velocity (Vmax) in the absence of any change in the Michaelis constant, Km, for transport (19,37). The two

parameters contributing to Vmax include 1) changes in the number of glucose transporters operating in the plasma membrane, 2) intrinsic activity of transporters, defined as the kinetic turnover number divided by Km (16). The number of glucose transporters operating in the plasma membrane can be altered by changes in the biosynthetic rate of the transporters, degradation rate of the transporters, or a redistribution between the plasma 0000membrane and a pool of latent inaccessible pre-existing transporters (59,64,69). The redistribution mechanism is found predominantly in insulin-sensitive tissues, e.g. adipose tissue. The intrinsic activity is a function of the turnover number of the carrier protein working on the membrane surface i.e the number of times per second the carrier can complete a transport cycle. This mechanism may be involved in the response to experimental hyperglycaemia, whereby a rapid increase in glucose transport across the intestinal BLM is observed without any change in the number of GLUT2 carriers present (73).

Diamond et al. postulated that the response of D-glucose uptake to switching from low to high carbohydrate diet involves increased expression of glucose transporters. To prove this hypothesis, specific binding of phlorizin to the intact intestinal mucosa was measured. The phlorizin binding was 1.9 times greater in jejuna of high-carbohydrate fed mice than of no-carbohydrate fed mice. The ratio reflected proportionate changes in average D-glucose uptake values in upper and lower jejunal sleeves, which were 9.1 ± 1.1 and $4.8 \pm .5$ nmol/ng.min in high carbohydrate and no carbohydrate mice respectively (37).

It was also demonstrated by the same group that the normal positional gradient in D-glucose uptake along the intestine arises from a gradient of existing transporters. By using the specific phlorizin binding approach, the glucose transport site density was 3.3 and 3.7 fold higher in jejunum and duodenum respectively, than in ileum (37). In addition, this gradient in glucose transport disappeared in mice on a carbohydrate free diet, with duodenal and ileal uptakes declining to the low level normally observed in the ileum. This led to the conclusion that the normal gradient of carrier expression along the small intestine was induced by the prevalent lumenal glucose concentration (63).

Ferraris and colleagues showed that the intestinal brush-border glucose uptake was up-regulated in response to a high glucose diet by increasing the numbers of transporters along the whole crypt-villus axis (39). The group identified a high-affinity phlorizin inhibitable binding site predominating from villus tip to midvillus and a site of possibly lower affinity predominating in the crypts. A similar response was observed with streptozocin-induced diabetic rats. Fedorak and colleagues demonstrated a 10 fold increase in density of phlorizin binding sites in the upper villus region of chronic diabetic rats (the region normally transporting glucose). In addition, recruitment of phlorizin binding sites was observed in the ileum occurring in both the upper and mid to lower villus region, (i.e. the portion not normally transporting glucose), (35).

The adaptive response of BLM transport to dietary carbohydrate has been found to be inducible within 3 days of switching from LC to HC diet and can be reversed within a similar period. Transport of glucose was measured across the BLM with isolated cells prepared from the whole small intestine or using purified basolateral membrane vesicles from jejunal scrapings. The maximal transport capacity was upregulated 2.4 fold with vesicle preps, in parallel with the increase in the number of cytochalasin-B binding sites when animals were put on the HC diet (39.7±2.1 pmol/ mg protein for HC diet and vs. 21.6±3.1 pmol/ mg protein for LC diet), (19).

I.3.2.1 Transcriptional Control

In the eucaryotic cell, transcription involves the copying, within the nucleus, of genetic information in the form of a linear sequence of nucleotides in DNA into a complementary strand of RNA. The primary RNA transcript is spliced to remove intron sequences, producing an mRNA molecule which is transported into the cytoplasm for subsequent protein synthesis (1). Control at the transcriptional level involves up- or down-regulation of the gene expressed by a combined effect of positive and negative gene regulatory proteins. For intestinal proteins, pretranslational mechanisms have been reported to involve changes in mRNA

transcription and/or degradation. A good example would be the post-natal regulation of intestinal lactase-phlorizin hydrolase (LPH) in humans. The enzymatic protein appears to be regulated at the level of transcription in a few selective groups of the human population. This was demonstrated by showing exact correlation between high levels of lactase mRNA and high levels of lactase enzyme expression (74). Other examples of intestinal proteins which exhibit changes during post-natal development and are mediated by pretranslational regulation include; fatty acid binding proteins (90), intrinsic factor (30), intestinal alkaline phosphatase (2), and calcium binding proteins (34).

I.3.2.2 Translational Control

Translational events appear to be very important in the adaptation of the enterocyte to the massive influx of nutrients during the course of a meal. These mechanisms serve the rapid up-regulation which occurs in response to a high concentration of dietary nutrients. For instance, lipoprotein levels including Apo AI and Apo AIV levels are increased in response to a meal via up-regulated protein synthesis without the increase of the level of mRNA encoding these proteins (46).

Post-transcriptional regulation has also been inferred for the expression of lactase during the post-natal development of the rat intestine, as lactase mRNA remained elevated long after the amount of active protein had declined (33,43). Expression of SGLT1 is also found to be controlled primarily at the level of post-transcription, during the post-natal development of the sheep intestine. It was demonstrated with the onset of weaning that the SGLT1 transport activity was 4-5 fold lower in vesicles prepared from the proximal intestine of 35 day old lambs compared to 14 day old animals, while SGLT1 mRNA remained the same as the animals matured (42).

Smith and colleagues also proposed a post-transcriptional mechanism for SGLT1 glucose transporter in rabbit jejunal enterocytes. They have demonstrated using in situ hybridization that SGLT1 mRNA remains elevated along the whole

villus axis but the glucose transport activity was confined to the upper two-thirds of the jejunal villi. It is suggested that the overall high levels of SGLT1 mRNA does not truly indicate the process by which the production of active SGLT1 occurs (93).

I.3.2.3 Post-translational control

Enterocytes may also utilize post-translational events to control expression of certain intestinal proteins by altering their rates of secretion and/or their half-life (via degradation). Rat skeletal muscle cells express both GLUT1 and GLUT4 transporters, however, the latter is translocated from a distinct intracellular organelle, known as the "glucosome" or "tubulovesicles" to the plasma membrane (92). These can be isolated and shown to be discrete from transverse tubules, plasma membranes, or sarcoplasmic markers. This translocation occurs in response to acute stimulation by insulin, exercise or hypoxia, and results in increased transport capacity because of the newly inserted glucose carriers now present at the cell surface (68,70).

Inhibition of oxidative metabolism in Clone 9 cells, a rat liver cell line, demonstrated biphasic enhancement of glucose transport. During the early phase of the response (0-2 h), the increase in glucose transport capacity was exclusively mediated by post-translational mechanisms without any change in cell GLUT1 protein or GLUT1 mRNA content. In contrast, the late phase of the response (8-24h) was characterized by pretranslational regulatory mechanisms associated with increases in GLUT1 mRNA and GLUT1 carrier protein (91).

I.4 Molecular Biology of Na^+-dependent and Facilitative Glucose Transporters.

In mammalian cells, there are two distinct families of glucose carriers which can be differentiated by their genetic makeup, primary structure and functional characteristics (94). The diversity exhibited among the glucose transporters expressed presumably reflects the importance of glucose as a source of metabolic energy (27). Hence, the different glucose transporters have evolved and represent the most efficient combination for delivery of glucose across epithelia or uptake by specific tissues to satisfy metabolic needs (98). For instance, glucose is absorbed by Na^+-

dependent active transport from the lumen of the small intestine and proximal nephron. Glucose is then transferred down its concentration gradient across the BLM via GLUT2, a facilitative glucose transporter. Subsequent glucose uptake by other tissues and cells is mediated by facilitative transporters whose properties are suited to the specific requirements of those tissues and cells for glucose and their role in the maintenance of glucose homeostasis.

The first class of glucose transporter sequenced using the Xenopus oocyte expression system was the sodium driven glucose cotransporter (54). Wright and co-workers isolated the cDNA clones expressed in rabbit small intestinal mucosa by carrying out injection of intestine poly (A)$^+$ RNA and measurement of alpha-methyl-D-glucopyranoside uptake in oocytes, a substrate which is specific for the Na^+/glucose transporter and is not transported by the GLUT facilitative transporter proteins. The sequence has been elucidated from the rabbit (54) and human (52) small intestine cDNA clones, sharing 85% identity in its coding sequence. The proteins are 662 and 664 amino acids in length for the rabbit and human respectively. The gene for human SGLT1 has been localized to the q11.2 qter region of chromosome 22 (53). The predicted secondary structure of SGLT 1 is an integral membrane protein with 12 membrane spanning domains and the amino and carboxy terminal located on the cytoplasmic side of the cell membrane (107). This protein is expressed in the BBM of mature enterocytes covering the intestinal microvillus tip and in the brush border membrane of proximal tubular cells of the kidney nephron (24,88). The transporter actively pumps glucose from the lumen of the intestine against its concentration gradient by coupling the transport of glucose with that of sodium (83). The electrochemical gradient for sodium across the BBM is maintained by a membrane bound Na^+/K^+ATPase, which pumps Na^+ across the basolateral surface of the epithelial cells (77). In addition to mediating active dietary glucose uptake, the carrier is also responsible for the reabsorption of filtered glucose in the proximal tubule of kidney (24).

It has been demonstrated that glucose/galactose malabsorption syndrome, a rare autosomal recessive disorder is caused by a mutation in the Na^+/glucose

cotransporter, SGLT1. The disease is characterized by neonatal onset of severe, watery, acidic diarrhea which leads to dehydration and the inability of the intestinal mucosa to take up glucose. It has been determined that a mutation of Asp28 to Asn28 in the human SGLT1 gene with glucose/galactose malabsorption syndrome results in complete loss of Na^+-dependent glucose transport (102).

In contrast to the SGLT1, a second type of glucose carrier is the family of facilitative glucose transporters (GLUT). These proteins transport glucose by a process which is saturable, bidirectional and passive i.e glucose crosses the cell membrane down its concentration gradient (49). These integral membrane proteins are expressed in nearly all mammalian cells. The cloning of 'GLUT' transporters was initially made possible when antibodies were produced against purified human erythrocyte band 4.5, the erythrocyte glucose transporter (104). These were then used to identify a complementary DNA clone for the glucose transporter from cDNA libraries constructed from a human hepatoma cell line HepG2 and rat brain (7,75). The 'GLUT' family is composed of structurally related proteins but are encoded by distinct genes that are preferentially expressed in certain tissues. To date, six facilitative glucose transporter genes (GLUT1,2,3,4,5,7) and one pseudogene (GLUT6) have been identified (44,45,65,66,75).

The 'GLUT' carriers share 39-64% in DNA homology and 50-70% identity in amino acid sequence (13). Although the 3-D structure of the transporter has not been elucidated, a model was proposed based on hydrophobicity plots, structural studies and proteolytic digests (75). These transporter proteins are predicted to span the cell membrane 12 times in the form of hydrophobic alpha-helices with both the amino and carboxyl termini, and the central region between helices 6 and 7 located on the cytoplasmic side of the lipid bilayer. Tryptic cleavage and sequence-specific antibody binding experiments have so far confirmed this predicted structure (15,51). Two systems of nomenclature have been used for the facilitated glucose carrier family. The numerical 'GLUT' designation signifies the chronological order in which these transporters were cloned or a name has been assigned to each isoform which is based on its major site or sites of expression (27). However, both of these

nomenclatures may be an oversimplification because, in most cases, identity between tissues has only been made on the basis of probe hybridisation to mRNA or recognition of only one small domain of the protein with antibodies.

The Xenopus laevis assay system has been used to over-express individual GLUT's to allow for characterization of functional properties such as substrate specificity and kinetic parameters (6,48,50,65,67,84,103), but not all isoforms insert properly which in part may be intrinsic to the transporter rather than the expression construct. For instance, it appears injection of GLUT 1, 2, 3 mRNA always stimulates glucose transport to a greater degree than injection of GLUT4. The lower activity of GLUT4 in this system may be a consequence of the predominant localization of GLUT4 to an intracellular compartment within the oocyte (50). Normally, GLUT4 expression in adipocytes and muscle cells is effected by translocation of the carrier proteins present in an intracellular pool, moving to the plasma membrane following insulin stimulation.

I.4.1 GLUT2 Liver Isoform

Complementary DNA encoding the human and rat brain glucose transporter (GLUT1) was used to clone a novel glucose transporter, (GLUT2) from a human and rat liver cDNA library using low stringency cross-hybridisation (44,97). A mouse cDNA encoding liver GLUT2 has also been isolated (3). B. Thorens et al. were able to confirm by expression in an *E. Coli* mutant, defective in glucose uptake, that the isolated clone from the rat liver cDNA library was indeed a glucose carrier. The protein was successfully incorporated into the bacterial membrane and the glucose uptake was stereospecific and inhibited by phloretin, a facilitative glucose transporter inhibitor (97). Rat GLUT2 shares 55% homology in the amino acid sequence to GLUT1, the rat brain glucose transporter, and most likely has the same structural orientation since the hydropathy profiles of the two GLUT proteins were virtually superimposible despite a 45% difference in sequence. It has been suggested that the transmembrane alpha helical segments 5 and 10 are involved in glucose binding since the region is quite conserved and shares an identity of 75-80% between GLUT1 and

GLUT2. However, the exoplasmic loops between transmembrane domains 1 and 2 are completely different, both in length and sequence (97). There is only 81% identity between the sequence of human and rat GLUT2 with most divergence occurring in regions at the large extracelluar loop and intracellular C-terminal domains (13).

Northern blot analysis showed liver glucose transporter message was distributed in tissues of liver, intestine, kidney and in the endocrine cells of the islets of Langerhans (97). Further immunofluorescence studies have confirmed and localized GLUT2 to the insulin producing beta cells of pancreatic islets (79), sinusoidal plasma membrane of hepatocytes (99), the basolateral membrane of proximal tubule cells of kidney (96) and the basolateral membrane of absorptive epithelial cells of the small intestine (79). However, western blot analysis detected size differences between the proteins expressed in different tissues. GLUT2 carrier of 53 kDa was determined in the liver, 61 kDa in intestine, 57 kDa in kidney and 55 kDa in pancreatic islets (97,99).

The kinetic characteristics of GLUT2 were determined in order to evaluate the contribution of the isoform to the maintenance of glucose homeostasis. The two kinetic parameters which determine the transport rate are Vmax, maximal transport velocity, and Km, which inversely reflects the affinity for glucose. Kinetic studies of the GLUT2 transporter revealed a significant difference in its capacity to transport glucose in comparison to GLUT's 1,3 & 4. GLUT2 has a Km of 20~66mM (50,106), approximately 10 fold higher than other isoforms. Using the Xenopus laevis assay system, GLUT2 has been characterized with 3-O-methylglucose as having a Km of 42.3±4.1 and 66 mM for D-glucose (50). This suggests GLUT2 transport capacity can increase proportionally with increases in extracellular or intracellular glucose over a range of physiological glucose concentration without becoming rate limiting (80). Hence, the significance of a high Km is to provide a rationale for its localization to tissues where major fluxes of sugars occur, e.g. small intestine. The intrinsic activity or the turnover number of the glucose transporter is also an important determinant of Vmax. However, the parameter is quite difficult to assess since many tissues

express more than one GLUT isoform (59).

Characterization of the exact role that GLUT2 plays has been recent. Gould and Bell (48) demonstrated via injection of GLUT2 mRNA in Xenopus oocyte that the carrier also facilitates the transport of fructose with a Km ~ 60mM. However, there are some concerns about how valid it is to extrapolate from this data to the expression of GLUT2 in other tissues. The liver GLUT2 protein also has a 10 fold lesser affinity for cytochalasin-B than other isoforms (4).

Detailed characterization of GLUT2 in the intestine BLM has shown that fructose and glucose can completely inhibit the transport of each other in isolated rat jejunal BLM vesicles (18). The Ki for fructose inhibition of glucose transport is 16mM. This strongly suggests that GLUT2 facilitates the efflux of fructose across the intestinal BLM. The clear difference in the affinity for fructose between liver and intestine and the fact that the Kd for CB binding to intestinal GLUT2 is 0.1 μM, compared to 1.0-1.8 μM reported for liver GLUT2, also raise the possibility that there are significant functional differences for GLUT2 depending upon the tissue in which it is expressed.

The islet GLUT2 cDNA coding region for the protein was sequenced in its entirety and found to be identical to that reported for liver (58,84). Thus, islet and liver forms of GLUT2 are produced from a single gene and identical mRNA. However, 3-O-methylglucose uptake into dispersed islet cells was inhibited by the presence of 1.0 μM cytochalasin-B, a concentration lower than that reported for hepatocytes. This may be due to size differences of the translated protein attributed to post-translational modification (58,97). Recent studies suggest a potential role for glucose as part of the "glucose sensing apparatus" to elicit insulin secretion. GLUT2 expression is decreased in B-cells from rats with experimental or spontaneous diabetes (80) but increased in rats made hyperglycaemic by glucose infusion (56), which suggests that expression of GLUT2 in islets plays an important role in the maintenance of blood glucose levels. It is speculated that perhaps GLUT2 is acting in tandem with glucokinase to regulate the control of insulin secretion from beta-cells (21).

The localization of liver GLUT2 to the sinusoidal membrane of hepatocytes and its absence from the bile canaliculi membrane domain suggest that it is involved in glucose uptake from blood as well as in release to the blood of glucose either stored as glycogen or produced by hepatic gluconeogensis (99).

Expression of GLUT2 in the basolateral membrane of the cells forming the S1 part of the kidney proximal tubule suggest that it is involved in the bulk reabsorption of glucose in the proximal convoluted tubules. Glucose reabsorption in the kidney is a two step process initiated by the Na^+-driven glucose symporter in the BBM of absorptive cells. These cells also express in their BLM, a high level of GLUT2 which is suited for the rapid rate of transepithelial reabsorption of glucose occurring in this part of the proximal tubule (96).

I.4.2 GLUT5 Intestinal Isoform

One of the most recent members of the GLUT gene family to be identified is GLUT5, the intestinal isoform. The cDNA clone was isolated from a human jejunal cDNA library by probing with human GLUT1 in low stringency cross hybridization (65). GLUT5 encodes a 501 amino acid polypeptide and shares 42,40,39, and 42% in homology with human GLUT's 1,2,3 & 4, respectively. It has been confirmed that the human GLUT5 gene is localized to the short arm of chromosome 1 in the region p32-p22. The structural orientation in the lipid bilayer is predicted to have twelve membrane spanning domains, similar to that proposed for GLUT1 and the other members of this gene family. The primary sequence consisted of 11 exons, compared to GLUT1, it has an additional intron subdividing exon 9 into 9A and 9B (27), but the functional significance of this difference is not clear.

GLUT5 is expressed primarily in the small intestine, with the majority localized toward the proximal region (duodenum and jejunum) coinciding with the predominant site of expression of GLUT2 and SGLT1. Immunocytochemical analysis has localized human GLUT5 to the brush-border surface of mature enterocytes and this was confirmed by western blot analysis of purified human jejunal brush border membrane preparation (26). In addition, GLUT5 is highly expressed in human

spermatozoa, and immunocytochemical studies suggest that the expression is restricted only to the most mature spermatocytes in the testis (12). In contrast to the multiple GLUT5 RNA transcripts found in the intestine, only the 2.8 kb transcript is found in testis. Western blotting has demonstrated the presence of a 48 kDa protein in testis, and two proteins of 48 kDa and 50 kDa in ejaculate spermatozoa (12). It is speculated that the larger of the GLUT5 proteins may be due to changes in glycosylation which occurs in membrane proteins of spermatozoa as they mature in the epididymis (10). A recent study by Shepherd et al (89) has shown that GLUT5 is also constitutively expressed in other tissues, including post-nuclear membranes of kidney, heart and four different skeletal muscle groups, brain, and in plasma membranes from adipocytes. This localization has been confirmed by western blot analysis and the immunoreactive bands detected have proven to be specific since the reaction disappears when the antibody is preincubated with the antigenic GLUT5 peptide. There appears to be some variation in the apparent molecular weight of the protein in different tissues (49,500-55,000 Mr) presumed to be due to varying glycosylation. Subsequent cleavage of the n-linked carbohydrate moiety with N-glycanase reduces the size of bands by 5,000-10,000 Mr. This results in bands of identical molecular mass (~45,000 Mr) in all tissues. The presence of GLUT5 in these tissues has also been confirmed by Northern blot analysis of total RNA (89).

Human intestinal GLUT5 was expressed in the Xenopus oocyte and its substrate specificity and kinetic properties determined (12). In this system the carrier exhibits high affinity (Km=6 mM) and selectivity for fructose. The human clone shows little capacity to transport glucose, whereas the rat clone can. In adipocytes, the high affinity fructose transport activity is not stimulated by insulin (89). However, the uptake of fructose in adipose cells, muscle, and brain is assumed to be mediated by GLUT5, since GLUT2 is not present in these tissues. The finding of GLUT5 on the mature spermatozoa seems to be consistent with the metabolic needs of these cells while remaining in seminal fluid which is rich in fructose. The human spermatozoa utilizes fructose initially and shifts to glucose when in the female reproductive tract (12).

The elucidation of the rat GLUT5 cDNA has recently been reported by Rand and workers. The rat GLUT5 cDNA was cloned by screening a rat jejunum cDNA library with human GLUT5 cDNA cross-hybridization. The coding region shared a homology of 87.3% in nucleotide and 81.5% in amino acid sequence which is the extent of sequence divergence often seen in comparison of cognate human and rat proteins (86). However, tissue distribution studies clearly demonstrated that rat GLUT5 was not detected in rat adipose, skeletal, muscle and testicular tissues, whereas the human GLUT5 message has been shown to be present in these tissues (89). It has been found that lactate was the primary hexose in seminal fluid in rats and most likely is the preferred energy substrate for metabolism of rat spermatocytes instead of fructose (86). Another characteristic which differentiates rat GLUT5 from that of humans is its ability to transport glucose as well as fructose. Expression of synthetic rat GLUT5 mRNA in Xenopus oocytes showed that rat GLUT5 was able to mediate glucose uptake to a lesser extent than fructose since [^{14}C]-fructose uptake was inhibited by addition of 50 mM glucose (86). This implies that the uptake of dietary glucose in the rat small intestine may occur both by secondary active transport via SGLT1 and by facilitative diffusion via GLUT5.

It is assumed from the above evidence that in the intestine GLUT5 facilitates uptake of fructose from the lumen across the brush-border membrane. The GLUT2 will then mediate the efflux of fructose from these cells across the BLM since there is little if any GLUT5 present in the basolateral membrane.

A. OBJECTIVE

To investigate the mechanistic basis of intestinal adaptation to changes in dietary carbohydrate.

B. PRIMARY GOALS

1) GLUT2:

-to characterize regional expression of GLUT2 mRNA along the small intestine by Northern blot hybridization analysis.

-to quantitate GLUT2 mRNA and protein levels in response to high/low carbohydrate diet by employing Northern and western blot analysis.

-to establish a correlation between GLUT2 mRNA and protein levels in search for possible transcriptional and/or post-transcriptional regulation.

2) GLUT5:

-to localize intestinal GLUT5 transporter protein using immunocytochemistry.

-to characterize regional expression of GLUT5 mRNA along the small intestine by Northern blot hybridization analysis.

-to examine GLUT5 mRNA expression in response to high/low carbohydrate diet.

3) To describe the molecular mechanisms which up-regulate intestinal facilitative glucose transport in response to high carbohydrate diet.

C. RATIONALE

goal #1

Transcription can play a paramount part in controlling gene expression by up- or down-regulating mRNA levels within the cell or by modulating the rate of mRNA production. By measuring the steady-state level of mRNA one may be able to deduce whether gene transcription is the primary mechanism in controlling expression of the gene product. Thus, it is essential to establish first the abundance and pattern of GLUT2 mRNA expression along the different regions of the small intestine at the basal level. Any modulation in the level of mRNA in response to diet can then be detected and compared to that present at the basal level.

goal #2

Tissue distribution studies indicate that GLUT5 mRNA is predominantly expressed in the intestinal tissue. In 1990, when this study was undertaken, it was not yet known whether GLUT5 protein carrier was involved in the uptake of dietary sugars across the luminal surface or mediated their release across the basolateral membrane of absorptive epithelial cells and into the interstitium. Hence, localization of GLUT5 would define at which pole of the enterocyte the carrier participates in the transcellular movement of hexoses. Initially, the abundance and pattern of GLUT5 mRNA expression along the small intestine will be clarified by northern blots, before the dietary treatment experiments are undertaken.

goal #3

Dietary adaptation in animals fed high carbohydrate diets is associated with increased glucose transport capacity. This is likely achieved by changes in transporter biosynthesis or stability. By measuring the GLUT2 mRNA levels in correspondence to its gene product, one could elucidate the molecular basis which controls GLUT2 carrier expression i.e. by transcriptional and/or post-transcriptional mechanisms.

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II. POST-TRANSCRIPTIONAL CONTROL OF INTESTINAL GLUT2 IN DIETARY ADAPTATION

II.1 INTRODUCTION

Intestinal absorptive epithelial cells exhibit the remarkable ability to adapt to high loads of carbohydrate over a relatively short period of time. Numerous studies have demonstrated that this intestinal adaptation requires alteration in the glucose transport capacity both in the brush border (SGLT1) and basolateral membranes (GLUT2) of the enterocyte (3,6,10,12). However, the underlying controlling molecular mechanism responsible for this change remains to be determined. Detailed kinetic studies from our laboratory have demonstrated that the basolateral membrane glucose transport (GLUT2) can be induced within 3 days of switching from low to high carbohydrate diet (LC, HC) and the induction is maintained for as long as the duration of the HC feedings. This apparent increase in the activity of BLM glucose transport is characterized by an increasing Vmax, and an increased number of cytochalasin-B inhibitable glucose carriers in the cell membrane while the affinity of the carrier, Km, is not affected. This is unlike the rapid type of regulation seen in response to acute hyperglycaemia (7). Based on these studies, we employed Northern and western blot analyses to investigate the mechanisms which the small intestine utilizes to up-regulate BLM GLUT2 carriers in response to increases in dietary carbohydrate.

In the present study, we first characterized the intestinal distribution of GLUT2 mRNA and secondly, looked for a correlation between GLUT2 mRNA and protein expressed during dietary adaptation in rats.

II.2 MATERIALS and METHODS

Animals and Dietary Treatment. Male Sprague Dawley rats weighing approximately 250 g each at six weeks of age were used for all diet experiments. Fifteen rats housed three to a cage were fed *ad libitum* two carbohydrate diets of different concentration. The rats were fed a low carbohydrate (LC) diet for 7 days

and were then switched to a high carbohydrate (HC) diet for the subsequent 7 days followed by another 7 days of LC diet. The total dietary treatment took three weeks and the rats were killed on days 7,8,10,14,21. Scrapings of intestinal mucosa were used to prepare total cellular RNA. For basolateral membrane vesicles preparations, twenty-four rats housed 3 to a cage were fed *ad libitum* the low carbohydrate (LC) diet for 7 days and switched to the high carbohydrate (HC) diet for 9 days. The rats were sacrificed on days 7,8,9,10,12,& 16, and four rats were used per data point.

Diets. The two diets were made isocaloric by altering reciprocally the fat and carbohydrate content while the protein was kept constant to minimize non-specific changes to the intestinal morphology. Previous studies have shown that rats fed these two diets maintain a normal food intake and weight gain after an initial increase because of the novelty of the food. This only lasts two to three days. The low carbohydrate rat chow consisted of 26.67% fat (ratio 42:56.4:1.6 of stearine, safflower oil, linseed oil respectively); 29.25% protein (casein); 23% glucose (dextrose); 5.53% mineral mix; 1.1% vitamin mix; 0.43% choline-Cl; 0.67% I-inositol; 0.28% L-methionine; 7.68% cornstarch; 5.39% non-nutritional cellulose. The high carbohydrate rat chow consisted of 6.49% fat (42.7% stearine, 52.7% safflower oil, and 5.3% linseed oil); 23.74% protein; 44.24% glucose; 4.49% mineral mix; 0.89% vitamin mix; 0.35% choline-Cl; 0.54% I-inositol; 0.23% L-methionine; 14.75% corn starch; 4.28% non-nutritional cellulose (6).

Intestinal tissue and RNA isolation. To facilitate total RNA extraction, intestinal mucosal scrapings were pulverized under liquid nitrogen and homogenized in 4M guanidine isothiocyanate. The homogenate was subsequently layered onto a 5.7M cesium chloride cushion and centrifuged in a SW-41 Ti rotor for 24 hours at 30 000 rpm, 20°C (8). Polyadenylated RNA was isolated from total RNA using oligo-(dT) cellulose chromatography (1).

GLUT2 cDNA. Probe specific for the human GLUT2/liver isoform glucose transporter: GLUT2/pGEM4Z-HTL3 was kindly provided by Dr. Graeme Bell, University of Chicago. The plasmid containing the 2.4 kb GLUT2 insert was transformed into DH5αF' which allows screening of positive recombinants by testing

for functional expression of a β-galactosidase gene. In vitro transcription generated radiolabelled antisense [^{32}P]-CTP RNA fragments from linearized plasmid DNA using T7 RNA polymerase (Riboprobe Gemini System, Promega).

Northern hybridization. RNA was size fractionated through 1% agarose/2.2M formaldehyde gel for 8 hours at 50 volts and then transferred onto a nylon membrane via capillary action. The RNA was fixed by baking the membrane in a vacuum oven for 2 h at 80°C. Initial prehybridization was carried out at 50°C for 1.5 h in a solution composed of 60%(v/v)formamide, 1X SSPE (180mM NaCl, 10mM $NaHPO_4$(pH 7.4), 1mM EDTA), 0.5% blotto, 10%(w/v) dextran sulfate, 1%(w/v) SDS, and 500 μg denatured salmon testes DNA/ml. Antisense probes ($2x10^6$ d.p.m./ml) were added to fresh solution and hybridization was carried out for 16 h at 50°C. Membranes were rinsed at room temperature in 2x SSC (1x SSC is 150mM NaCl, 15mM trisodium citrate, pH 7.0), washed in 2x SSC, 0.1% SDS for 15 min at RT, washed in 0.2x SSC, 1% SDS for 15 min at 70°C and then rinsed in 0.2x SSC. The integrity and accuracy of loading RNA onto the gels were confirmed by examining ethidium bromide staining of the 18S and 28S ribosomal RNA bands under UV illumination, (integrity of sample RNA can be assessed by the ratio of 28S/18S rRNA levels, which should be close to 2.0. A lower ratio reflects partial degradation of 28S rRNA band, largely due to the susceptibility of the larger RNA species to degradation). Autoradiography was carried out at -70°C with an intensifying screen using Kodak XAR5 X-ray films. All autoradiograms were scanned by LKB Ultrascan XL densitometer to make relative comparisons.

Control measures for Northern Blot Analysis. 30 μg of total RNA from normal duodenum, jejunum, ileum and colon were loaded for every Northern blot in an attempt to make the subsequent hybridization comparable from one blot to the next. To correct for possible differences in the loading of mRNA onto the lanes or transfer over from the gel onto the nylon membrane, the blots were stripped of GLUT2 cRNA probe by washing twice in 0.1x SSC, 0.5% SDS for 20 minutes at 95°C. They were then reprobed using cRNA fragments for gamma-actin, a constitutively expressed cellular element which was assumed to be unaffected by the diets.

Quantification of Northern hybridisation. The gamma-actin radiolabelled membranes were again exposed to XAR5-X film and the resulting autoradiograms scanned as before. All quantification of the Northerns were then normalized on each film to the gamma-actin level for 1 week of LC feeding. Thus each film gave a set of densities for each lane which were normalised against the first sample from the same tissue.

Preparation of basolateral membrane vesicles and western blotting. Rat jejunal and ileal basolateral membrane vesicles (BLV) were prepared from mucosal scrapings as described by Maenz and Cheeseman (17). The jejunum, measured 12-43 cm from the pylorus and the ileum, 45-95 cm were excised and flushed with ice cold phosphate-buffered saline (pH 7.4) containing 0.1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The mucosal scrapings were subsequently homogenized in ice cold sucrose buffer (250 mM sucrose, 2 mM Tris-HCl pH 7.4, 0.1 mM PMSF) using a polytron for 3X30 second pulses at 4°C. The homogenate was centrifuged at 4,750 rpm for 15 min and the supernatant was saved and centrifuged at 13,100 rpm for 20 min. The resulting upper white fluffy pellet was resuspended in sucrose buffer without disturbing the lower brown pellet and subjected to glass-Teflon homogenization (8 strokes). The homogenate was brought up to a final volume of 35 ml with sucrose buffer containing a final concentration of 12% v/v Percoll. Centrifugation followed at 20,000 rpm for 1.0 h until a Percoll density gradient was obtained. The initial 9 fractions were collected (42 drops per fraction) starting from the top of the gradient using an automated Gilson fraction collector. By performing Na^+/K^+ ATPase enzyme assays as described by Parkinson et al (20), fractions 4,5,6 were identified to contain the enriched basolateral membranes. The BLV were resuspended in buffer (125 mM potassium thiocyanate, 2 mM Tris-HCl, pH 7.4) and protein concentration was determined by the Biorad protein assay (Biorad), using BSA as a standard. Basolateral membranes (15 μg) were solubilized in Laemmli sample buffer (16) and fractionated through 10% sodium dodecyl sulphate-polyacrylamide gel using Mini-PROTEAN II cell (Biorad). The proteins were immobilized onto nitrocellulose membrane (Biorad) by electrotransfer for 1 h

15 min at 4°C using the Mini Trans-Blot Cell (Biorad). The membrane was stained for total protein with ponceau S to ensure equal amounts of protein were loaded. Blocking of the membrane was carried out in 3% nonfat dry milk in TPBS (0.05% Tween 20, phosphate-buffered saline, pH 7.4) for 1 h and then incubated with 1:1 000 rabbit polyclonal antibody to rat GLUT2 (East-Acres Biologicals, MA) in 3% nonfat dry milk in TPBS overnight at 4°C. The membrane was washed three times in 3% nonfat dry milk/TPBS for 15 min, 1 h, 15 min, respectively, followed by incubation with 1 μci of ^{125}I-protein A (Amersham) in 3% nonfat dry milk/TPBS for 1 h. Three subsequent washes followed as described above. The membrane was rinsed briefly with PBS before autoradiography using Kodak XAR-5 film with an intensifying screen at -70°C.

II.3 RESULTS

Intestinal GLUT2 mRNA distribution. Rat intestinal polyadenylated RNA was isolated from the duodenum, jejunum, ileum and liver (positive control). A 2.8 kb mature transcript encoding the rat GLUT2 gene was detected in all regions (fig.II.1), except colon (fig.II.2). The majority of the GLUT2 mRNA is expressed in the jejunum. According to densitometer readings, the relative abundance of jejunal GLUT2 mRNA exceeds those of duodenum and ileum by 6.8 and 5.3 fold respectively.

GLUT2 expression in response to diets. Messenger RNA levels: The animals were fed low carbohydrate diet for 7 days before switching to HC diets. Both duodenum and jejunum appeared to have a similar pattern of response characterized by a transient increase of GLUT2 mRNA after initial 24 h of HC feeding, followed by eventual down-regulation of the gene by day 7 (fig.II.2). The duodenum showed a rapid 314% increase in the mRNA levels, which was maintained until day 3, after which the levels decline below that of control (table II.1). Jejunal GLUT2 mRNA increased 212% (~2 fold) after a 1 day HC feeding, and was then down-regulated to 127% by day 3 and below that of control by day 7 (table II.1). In contrast, ileal mRNA levels increased 666% (~7 fold) with 1 day of HC diet and continued to

increase to 1316% (~13 folds) by day 7 of HC feedings (Fig.II.2, table II.1).

Protein levels. Western blotting of basolateral membrane vesicles using a polyclonal antibody to rat GLUT2 detected a 61 kDa protein (fig.II.3), consistent with previous reports by Thoren et al. (23). Fig.II.4 and table II.2 show that GLUT2 carrier expression increased dramatically 3 fold by Day 10 of HC feeding.

Internal standard. All Northern blots from the dietary treatment experiments were stripped of GLUT2 anti-sense RNA probes and rehybridized to a human gamma-actin probe correlated 270 base pair of C-terminal coding region, 130 base pair of 3' untranslated region and 130 base pair of vector sequence. Intensity of GLUT2 and gamma-actin mRNA bands was scanned and quantitated by the LKB densitometer (table II.1; absorbance in arbitrary units). All HC treated GLUT2 mRNA levels (in discussion) were expressed as a percentage of mRNA levels from the low dietary carbohydrate treated animals. To ensure hybridization consistency, 30 ug of total RNA from duodenum, jejunum, ileum and colon were loaded for every Northern blot.

II.4 DISCUSSION

The intestinal facilitative glucose transporter mediates the efflux of absorbed glucose from the enterocyte, participating in the transcellular movement of dietary glucose with the Na^+/glucose cotransporter. Studies from our laboratory and recent reports indicate that GLUT2 also facilitates the transport of fructose across the basolateral membrane of the small intestine (5,13). Cloned by B. Thorens et al., 1988, GLUT2 is a member of the facilitative glucose transporter supergene family (4,22). To date, the carrier has been localized to the insulin producing beta cells of pancreatic islets, sinusoidal plasma membrane of hepatocytes, the basolateral membrane of proximal tubule cells of kidney and absorptive epithelial cells of the small intestine (19,23,24). In the rat small intestine, the expression of GLUT2 mRNA is restricted to duodenum, jejunum and ileum (fig.II.2). The highest level was found in the jejunum (fig.II.1), corresponding to the primary site of glucose absorption (14). Secondly, I found a decline in mRNA level along the length of small

intestine which parallels the regional gradient in glucose transporter activity from duodenum to ileum (15). Within 24 h of HC feeding, I observed the jejunal GLUT2 mRNA to increase 2 fold. This increase parallels the change in basolateral glucose transport, Vmax and cytochalasin-B binding density previously demonstrated by Cheeseman and Harley (1990), who reported a corresponding increase of 2 fold by day 3 of HC feedings (6). However, the change of GLUT2 protein levels in response to the HC diet takes a day or more before a 2-3 fold increase was detected. This delay in response might be explained by the time required for the new population of enterocytes to migrate along the crypt-villus axis reaching the mid and upper region. Glucose transport is not fully expressed until the epithelial cells reach the midvillus region where they become mature and fully differentiated. So far, there has not been any study to indicate which enterocyte subpopulation (crypt, lower, mid, upper) along the crypt-villus axis giving rise to the increase of GLUT2 carrier. Recent reports by Ferraris and Diamond using phlorizin-binding studies have demonstrated that SGLT1, the sodium-dependent glucose carrier, density increased along the terminal villus and probably along the whole crypt-villus axis in response to HC diet (10). However, the signal for altering glucose transport was perceived in the crypt cells causing a delay in the uptake response which was largely due to cell migration time (the time required for the cells travelling from crypt to terminal region of the villus), (9,11). These findings might very well explain the delay in the increased GLUT2 carrier expression since the half life of an enterocyte is at least 18 h. Hence, it would require at least 1.5 days for the old enterocytes to slough off and be replaced by a new population of migrating cells expressing a higher glucose transporter density.

Throughout the rest of the HC feeding period, duodenal and jejunal mRNA levels declined progressively. The levels were dropped below that of control (LC) by day 7 which suggests transcription is not the primary mechanism in sustaining the high level of GLUT2 expression in dietary adaptation. Based on the lack of correlation between protein and mRNA levels, the GLUT2 protein expression must be regulated post-transcriptionally.

The down-regulation of GLUT2 mRNA poses some interesting questions and

needs to be the subject of further investigations. Questions which still need to be addressed include:

1) Is the decrease in GLUT2 mRNA level significant in maintaining the physiological range of glucose concentration allowed to enter the system by exerting control at the transcriptional level. Therefore, does down-regulation of the glucose transporter gene occur during prolonged periods of excess glucose to prevent overloading in the system? 2) Studies carried out by Asano et. al, demonstrated that GLUT2 mRNA was down-regulated in the presence of pyruvate, a metabolite of glycolysis (2). Could this hold true for enterocytes metabolizing the excess glucose available to its system and generating pyruvate metabolites which in turn down-regulates the gene? 3) Is the down-regulation of GLUT2 message a consequence of the experimental design of the diets? In other words, will transcription predominate if the increase in glucose content in HC/LC is maintained at a higher ratio. 4) Do different mechanisms get switched on depending upon the glucose concentration and what is the most efficient and energetically favorable mechanism for the system to accomodate the load. Answering some of these questions would give insight into the dynamics of regulation at the cellular level.

The normal luminal glucose concentration parallels the transport gradient along the small intestine where the maximal glucose concentration lies in jejunum, then declines steeply towards the ileum. The free glucose concentration in the duodenum is not as high as in the jejunum since the majority of complex sugars has not been hydrolysed completely by pancreatic and intestinal amylases at the entry of the small intestine. This is reflected in GLUT2 mRNA levels present in jejunum exceeding that of the duodenum by 6.8 fold. When the rat has been continuously fed high dietary carbohydrates, the distal intestine (ileum) becomes saturated with more unabsorbed glucose which is spilt over from the jejunum, thereby causing induction of ileal glucose transport (12). This is observed by the stimulation of ileal mRNA on day 1 of the HC feeding and throughout the HC feeding period. It is therefore conceivable that the ileum may serve as a compensatory tissue absorbing leftover free glucose which the duodenum and jejunum could not accommodate.

Figure II.1 Regional distribution of GLUT2 poly(A^+) RNA in rat small intestine. Poly(A^+) RNA (5 μg), isolated from normal rat liver (lane 1, positive control), duodenum (lane 2), jejunum (lane 3), ileum (lane 4), were hybridized with a 2.4 kb in vitro transcribed radiolabelled antisense[^{32}P]-CTP RNA probe, generated from XbaI linearized human GLUT2 cDNA in pGEM-4Z plasmid. The Northernblots were performed as described in the materials and methods (section II.2) and the autoradiogram shown was exposed for 24 h at -70°C with intensifying screens.

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Figure II.2 Regional expression of GLUT2 mRNA in response to low/high carbohydrate dietary treatment in rat small intestine and colon. Panel A: Northern blotting of total RNA (30 μg per lane) demonstrates the effects of 7 day LC feeding, followed by 1,3,7 day of HC feeding and finally, by 7 day of LC diet, from left to right, in duodenum (lane 1-5); jejunum (lane 6-10); ileum (lane 11 -14); colon (lane 16-19).(data of final 7 day LC diet for ileum and colon are not available).Northern blots were hybridized with [^{32}P]-CTP labelled antisense RNA probes, which were in vitro transcribed from XbaI linearized human GLUT2 cDNA in pGEM-4Z plasmid. The Northern blots (NT) were performed as described in the materials and methods (section II.2). The corresponding panel B were the same NT blots reprobed with human gamma-actin, which was used to normalize comparisons between lanes for GLUT2 RNA abundance.

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Figure II.3 Western blotting of GLUT2 in rat jejunum in response to low/high carbohydrate diet. Jejunal basolateral vesicles (BLV) were prepared as described under materials and methods (section II.2) and electrophoresed on 10% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose and immunoblotted with a COOH-terminal polyclonal anti-rat GLUT2 antibody. The membranes were developed with ^{125}I-protein A and autoradiographed for 48h at -70°C. The BLV were loaded (15 μg/lane) in an order following the time course of the diet (from left to right) and starting with 7 days of LC (lane 1), followed by 1,2,3,5,9 days of HC (lane 2,3,4,5,6).

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Figure II.4 The time course of induction of jejunal GLUT2 mRNA and transporter protein across the basolateral membrane from rat small intestine. Animals were maintained on the low carbohydrate diet for 7 days and then switched to the high carbohydrate diet 7 days for RNA extraction and 9 days for basolateral membrane isolation. The animals were then switched back to 7 days of LC diet for RNA extraction only.

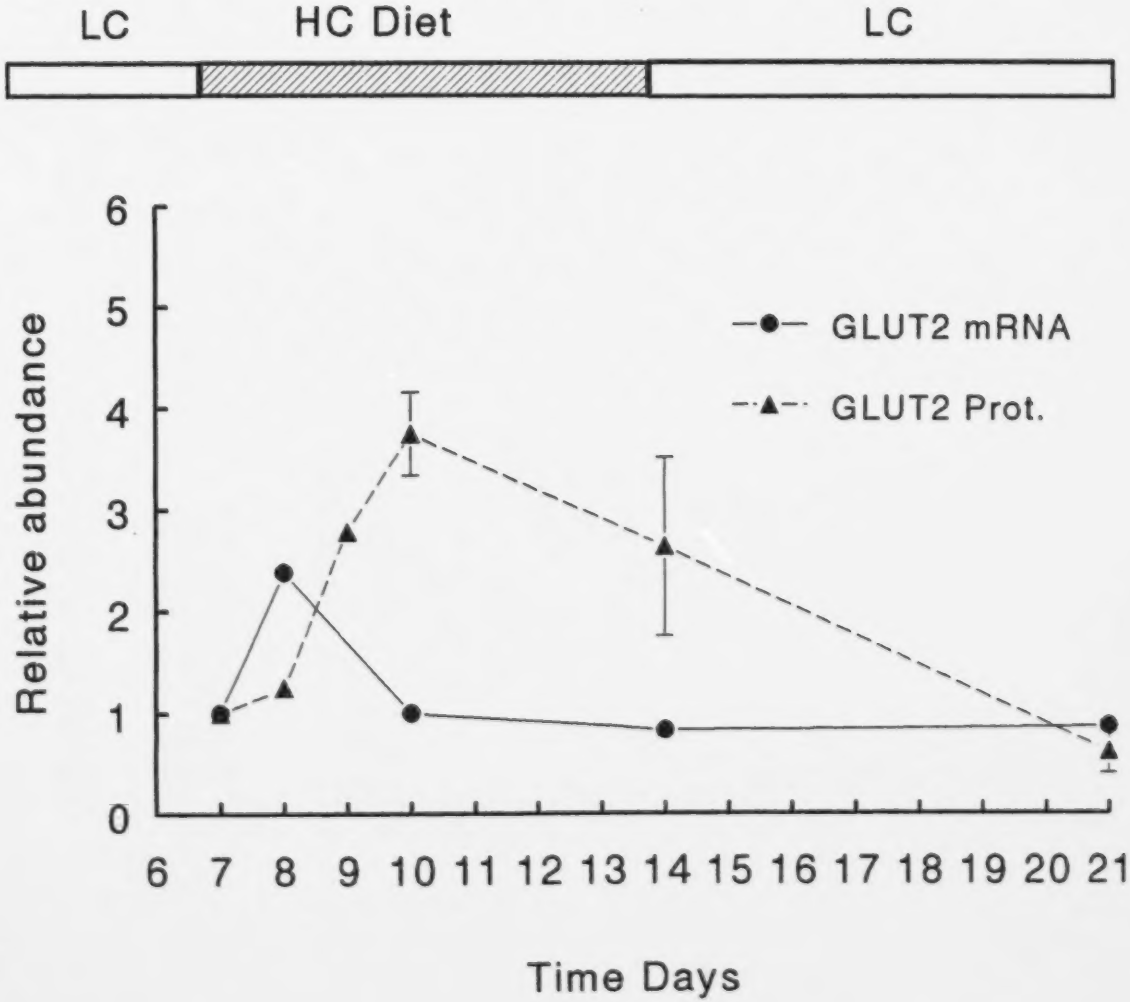
LC
HC Diet
LC
Relative abundance
0
1
2
3
4
5
6
GLUT2 mRNA
GLUT2 Prot.
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Time Days

Table II.1 The intestinal mRNA levels (absorbance, arbitrary units) for gamma-actin were normalised on each film to the gamma-actin level for 1 week of LC feeding in the same tissue. Thus each film gave a set of density for each lane which were normalized against the first sample of that particular tissue. The four group of tissue examined in response to LC/HC diet were duodenum, jejunum, ileum, colon and the normalised HC treated GLUT2 mRNA levels were than compared to that treated with LC diet.

Table II.1 Summary of GLUT2 mRNA levels in high/low carbohydrate diet experiment.

Tissue/Dietary Treatment	GLUT2 mRNA	Gamma-actin mRNA	GLUT2 mRNA normalized
duodenum 7LC	0.07	0.855	0.07
duodenum 1HC	0.43	1.692	0.22
duodenum 3HC	0.42	1.544	0.23
duodenum 7HC	0.02	0.511	0.03
duodenum 7LC	0.18	0.971	0.16
jejunum 7LC	3.08	5.674	3.08
jejunum 1HC	7.64	6.622	6.54
jejunum 3HC	4.13	5.964	3.92
jejunum 7HC	2.16	4.775	2.56
jejunum 7LC	3.32	5.968	3.16
ileum 7LC	0.06	3.007	0.06
ileum 1HC	0.13	0.999	0.40
ileum 3HC	0.82	4.842	0.51
ileum 7HC	1.12	4.274	0.79

Table II.2 Summary of basolateral GLUT2 protein in response to alterations in dietary carbohydrate.

	Jejunum	Ileum	
7 LC	1.0	1.0	7 LC
1 HC	1.25	8.0	1 HC
2 HC	2.78		
3 HC	2.93 ± 0.6	5.45 ± 3.76	3 HC
7 HC	2.33 ± 0.66	7.60 ± 3.7	7 HC
7 LC	0.65 ± 0.14	1.86 ± 0.07	7 LC

Table II.2 Summary of basolateral GLUT2 protein levels (absorbance: arbitrary units) in response to High/low carbohydrate diet.

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III. EFFECT OF HIGH DIETARY CARBOHYDRATE ON INTESTINAL FRUCTOSE TRANSPORTER, GLUT5

III.1 INTRODUCTION

The human intestinal GLUT5 transporter, cloned in 1990, is the most divergent member of the facilitative glucose transporter family, sharing only ~40% homology in its primary amino acid sequence with that of GLUT 1,2,3,4 (12). It was initially speculated that the function of the GLUT5 transporter was to participate in the uptake of dietary glucose across the brush border membrane. Recently it has been demonstrated that the key role it plays is as a fructose transporter, delivering dietary fructose across the epithelial brush border membrane of the small intestine. Expression of the protein by injecting mRNA into Xenopus oocytes showed that this carrier is a relatively high affinity fructose transporter (Km=6mM) (3). The subsequent efflux of fructose across the BLM is mediated by GLUT2 (10).

With an increasing fructose consumption in the western human diet, there is growing interest in the mechanistic basis of how fructose transport is regulated. Fructose has been shown to be incompletely absorbed in the small intestine, serving as a substrate for the colonic bacteria flora, which may lead to subsequent abdominal discomfort, diarrhea, and functional bowel syndrome (14). It has been found that the capacity for absorbing free fructose is limited in the human and rat (9). However, Solberg and Diamond exposed rats to long term dietary fructose and demonstrated adaptation of the intestinal transport system for fructose, ie an increase in its transport capacity. They have also shown that specific hexoses (glucose and galactose) can signal this change (18). Several studies have revealed that the movement of D-glucose across the intestinal epithelial cells not only affects its own transport but also exerts a mediating effect on fructose uptake (9,18). It has since been determined that glucose stimulates fructose absorption in a dose-dependent manner; it is only when fructose is present in excess of glucose that malabsorption

occurs (9). Thus, I set out to examine the stimulatory effect of glucose on GLUT5 gene expression by exposing rats to long term high dietary carbohydrate.

The aim of this study was to analyze first, the longitudinal distribution of GLUT5 transporter protein and its mRNA along the small intestine. Secondly, to characterize the regulatory pattern of GLUT5 mRNA transcript in response to high/low carbohydrate diets.

III.2 MATERIALS AND METHODS

Antibodies. Human GLUT5 polyclonal antibodies (BBG5) were raised against synthetic peptide corresponding to amino acids 492-501 of the human sequence carboxy terminus (KELPPVTSEQ) coupled to keyhole limpet haemocyanin. The decapeptide was synthesized as described (11) on an Applied Biosystem Model 430A peptide synthesizer by the Alberta Peptide Institute. Rabbits (Flemish Giant Dutch Lopped eared cross) were immunized intradermally with 500 μg of conjugated peptide emulsified in incomplete Freund's adjuvant. Subsequent boosts were carried out every 4 weeks for 5 months. Serum was then collected and checked for titre ($1x10^{-6}$ dilution was used).

Tissue preparation for immunocytochemistry. Sprague Dawley male rats, 6 weeks old and weighing approximately 250g were used for all immunocytochemical experiments. Collection of rat tissues was carried out while the rat was under surgical anesthesia (partial to complete loss of toe pinch withdrawal response). Somnotol (sodium pentobarbitol, 65 mg/ml) was injected intraperitoneally at 0.1 ml per 100g of the rat weight. The small intestine was excised from the ligament of Treitz to the ileocecal junction and further divided into three segments. The initial 12 cm extending from the ligament of Treitz was designated duodenum, the next 30 cm was the jejunum, and the last 50 cm was the ileum. Colonic tissue was collected and used for a negative control. All segments were flushed with phosphate-buffered saline containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and PLP fixative (2% paraformaldehyde in PBS, 75 mM lysine, and 10 mM periodate) (13), before everting the intestinal sleeves. Small mid-sections from each region were then dissected and

immersed in PLP for 18 hr at 4°C. Following rinsing in PBS, the tissues were pre-equilibrated in 0.6M sucrose in PBS overnight before embedding in OCT compound (Miles). Semiserial 10 μm sections were cut using a Minotone cryostat (IEC, Damon) and collected onto gelatin chromic potassium sulphate-coated slides. All slides were kept in PBS or frozen at -20°C before further processing.

Immunocytochemistry. The tissue sections were blocked for 20 min in PBS containing 1 % BSA (bovine serum albumin), then incubated at 4°C overnight with 1:50 dilution (in blocking buffer) with BBG5. Sections were washed three times for 5 min in PBS, then subjected to 1:75 dilution (in blocking buffer) of fluoresceinated goat anti-rabbit immunoglobulin G (Calbiochem). After final washes in PBS three times for 5 min, the sections were mounted in 60% glycerol, 2% n-propyl gallate, and 0.2 M Tris-HCL, pH 8.0. Sections were examined and photographed with a Leitz microscope using Kodak Tax 400 film.

Animals and Dietary Treatment. Fifteen male Sprague Dawley rats weighing approximately 250g each, at six weeks of age, were used for the diet experiment. The animals were housed 3 to a cage and were fed *ad libitum* a low carbohydrate (LC) diet for 7 days. The chow was then switched to a high carbohydrate (HC) diet for 7 days followed by another 7 days of LC diet. The dietary treatment took three weeks and the rats were killed on days 7,8,10,14,21. Scrapings of intestinal mucosa were taken for the preparation of total RNA.

Diets. The diets were isocaloric with the carbohydrate and fat content altering reciprocally while the protein content was kept constant. This approach minimizes alterations in food intake, weight gain and non-specific changes to the intestinal morphology. The low carbohydrate rat chow consisted of 26.67% fat (ratio 42:56.4:1.6 of stearine, safflower oil, linseed oil respectively); 29.25% protein (casein); 23% glucose (dextrose); 5.53% mineral mix; 1.1% vitamin mix; 0.43% choline-Cl; 0.67% I-inositol; 0.28% L-methionine; 7.68% cornstarch; 5.39% non-nutritional cellulose. The high carbohydrate rat chow consisted of 6.49% fat (42.7% stearine, 52.7% safflower oil, and 5.3% linseed oil); 23.74% protein; 44.24% glucose; 4.49% mineral mix; 0.89% vitamin mix; 0.35% choline-Cl; 0.54% I-

inositol; 0.23% L-methionine; 14.75% corn starch; 4.28% non-nutritional cellulose (4).

Intestinal tissue and RNA isolation. To facilitate total RNA extraction, intestinal mucosal scrapings were pulverized under liquid nitrogen and homogenized in 4 M guanidine isothiocyanate. The homogenate was subsequently layered onto a 5.7M cesium chloride cushion and centrifuged in a SW-41 Ti rotor for 24 hours at 30 000 rpm, 20°C (5). Polyadenylated RNA was isolated from total RNA using oligo(dT) cellulose chromatography (1).

GLUT5 cDNA. Probe specific for human GLUT5 intestinal glucose transporter: GLUT5/phJHT5 was kindly provided by Dr. Graeme Bell, Chicago. A 1.81 Kb BAMH1/ECOR1 cDNA fragment corresponding to the translated region was subcloned into the plasmid pGEM3 using low melting agarose electrophoresis and T_4 ligase at 10°C overnight. The GLUT5 containing plasmid was subsequently transformed into an *E. coli* derivative, HB101. Radiolabelled antisense [^{32}P]-CTP RNA fragments were transcribed in vitro from linearized plasmid DNA using T7 RNA polymerase (Riboprobe Gemini System, Promega).

Northern hybridization. RNA was size fractionated through 1% agarose/2.2M formaldehyde gel for 8 hours at 50 volts and subsequently transferred onto a nylon membrane via capillary action. The RNA was fixed by baking the membrane in a vacuum oven for 2.0 h. at 80°C. Initial prehybridization was carried out at 50°C for 1.5 h in a solution composed of 60%(v/v) formamide, 1X SSPE (180 mM NaCl,10mM $NaHPO_4$(pH 7.4),1 mM EDTA), 0.5% blotto, 10%(w/v) dextran sulfate, 1%(w/v) SDS, and 500 μg denatured salmon testes DNA/ml. Antisense probes ($2x10^6$ d.p.m./ml) were added to fresh solution and hybridization was carried out for 16 h at 50°C. Membranes were rinsed at room termperature in 2x SSC (1x SSC is 150 mM NaCl, 15 mM trisodium citrate, pH 7.0),washed in 2x SSC, 0.1% SDS for 15 min at RT, washed in 0.2x SSC, 1% SDS for 15 min at 70°C and then rinsed in 0.2x SSC. The integrity and accuracy of loading RNA onto the gel were confirmed by examining ethidium bromide staining of the 18S and 28S ribosomal RNA under UV illumination and reprobing the membranes with human gamma-actin. Autoradiography was

carried out at -70°C with an intensifying screen using Kodak XAR5 X-ray films. All autoradiograms were scanned by LKB Ultrascan XL densitometer to make relative quantitative comparisons.

Control measures for Northern Blot Analysis. 30 μg of total RNA from the duodenum, jejunum, ileum and colon were loaded for every Northern blot in an attempt to make the subsequent hybridization comparable from one blot to the next. However, after running the gels the mRNA was transferred over to a nylon membrane and it is difficult to ensure equal transfer for each lane. Therefore, it was necessary to use an internal control against which the GLUT5 bands could be normalised. This would also ensure equal amounts of sample were loaded for each lane.

Northerns from the dietary treatment experiments were stripped of the GLUT5 anti-sense RNA probes and rehybridized to a human gamma-actin probe correlating to 270 base pair of C-terminal coding region, 130 base pair of 3' untranslated region and 130 base pair of vector sequence. Gamma-actin is a constitutively expressed cellular element which was assumed to be unaffected by the diets. The blots were stripped by washing twice in 0.1x SSC, 0.5% SDS for 20 min at 95°C before reprobing with gamma-actin cRNA fragments.

III.3 RESULTS

Immunocytochemical Detection of Fructose Transporter. Polyclonal anti-serum BBG5 raised against the C-terminus peptide of cloned human GLUT5 fructose transporter recognized a protein localized to the brush border membrane of intestinal enterocytes in the rat (fig.III.1). Detected by FITC-conjugated secondary antibodies, I was able to show increasing immunofluorescent staining up the crypt-villus axis with no apparent staining in the crypt cells. The intensity gradient suggested the population of fructose transporters was primarily localized to the terminal portion of the villus (fig.III.2). The longitudinal expression of GLUT5 was found in duodenal, jejunal and ileal tissues with a decreasing staining gradient from the proximal region to the distal region of the small intestine. No apparent staining was found in the

colon (fig.III.3).

Intestinal distribution of GLUT5 mRNA. Polyadenylated RNA was isolated from duodenum, jejunum and ileum of the rat intestine and hybridized to a 1.81 kb in vitro transcribed anti-sense RNA probe to human GLUT5 cDNA (fig.III.4). The majority of GLUT5 mRNA was distributed along the jejunum and exceeded that of the duodenum and ileum by 2 and 3 fold respectively. GLUT5 message was not detected in the colon (fig.III.5). Northern blotting detected two mature transcripts of 2.0 and 2.8 kb encoding the rat GLUT5. Both transcripts were expressed in duodenum and jejunum but the 2.8 kb species was found predominantly in the duodenum and 2.0 kb largely in the jejunum. In the ileum, only the 2.0 kb transcript could be detected (fig.III.5). The physiological implication of this regional specificity is unclear.

Effect of dietary glucose on GLUT5 mRNA. The rats were fed low dietary carbohydrate (LC) for 7 days before switching the diet to high carbohydrate content (HC). After a 1 day feeding on HC diet, duodenal and jejunal GLUT5 mRNA showed a 44% and 30% increase respectively. Following 3 and 7 days of HC, jejunal GLUT5 mRNA levels fell progressively below that of the control. There was no such decline in the duodenal mRNA levels which remained high until day 7. Conversely, ileal mRNA increased 800% after a 1 day HC feeding and continued at high levels until day 7 (fig.III.5).

Quantification of Northern hybridisation. The gamma-actin radiolabelled membranes were again exposed to XAR5-X film and the resulting autoradiograms scanned as before. All quantification of the Northerns was then normalized on each film to the gamma-actin level for 1 week of LC feeding. Thus each film gave a set of densities (table III.1; absorbance in arbitrary units) for each lane which was normalised against the first sample from the same tissue. All HC treated GLUT5 mRNA levels were expressed as a percentage of mRNA levels from the low dietary carbohydrate treated animals.

III.4 DISCUSSION

Fructose is transported by a system not shared by any other dietary sugars. In vitro studies using everted intestinal sleeves by Schultz and Strecker (15) and kinetic analysis using intestinal brush border membrane vesicles by Sigrist-Nelson and Hopfer (17) demonstrated that fructose uptake occurs via facilitated diffusion and is mediated by a Na^+-independent carrier insensitive to phlorizin. GLUT5 was cloned by using previously identified 'GLUT' cDNA under low stringency hybridization conditions to identify and isolate mRNA coding for this protein. Initially it was assumed to be a glucose transporter although oocyte expression of the protein failed to show increased glucose transport. However, injection of synthetic human GLUT5 mRNA into Xenopus oocytes promoted fructose uptake. Kinetic characteristics have shown that it is a high affinity carrier (with a Km of 6 mM for fructose) which is not inhibited by 100 mM glucose or by 100 μM cytochalasin-B, a potent inhibitor of glucose carrier proteins (3). Recent distribution studies localized the GLUT5 transporter in a variety of human tissues, in particular the brush border membrane of the small intestine, the plasma membrane of mature spermatids. It is also found in low abundance in four different muscle group, as well as the kidney, heart, brain post-nuclear membranes and adipocyte plasma membranes (3,8,16).

My immunocytochemical localization of GLUT5 to rat duodenal, jejunal, and ileal enterocytes indicates that the transport of fructose probably occurs within these regions. The immunofluoresence showed an increasing staining of the enterocytes up the crypt-villus axis, suggesting there is increasing gradient of transport activity from crypt to villus tip. The abundance of the immunofluoresent stained carrier proteins is relatively consistent with the mRNA distribution of GLUT5 in the Northern hybridization analysis, showing high levels of GLUT5 in the proximal region and a gradual decline towards the distal portion of the small intestine. This pattern parallels the longitudinal distribution of SGLT1 (Na^+-glucose cotransporter) and GLUT2 (basolateral facilitative glucose transporter) in the small intestine. The distribution of these carriers is significant in that it reflects the luminal concentration gradient of their substrates. The fructose transporter is further characterized by two mature transcripts of 2.8 and 2.0 kb which were differentially distributed along

duodenal, jejunal and ileal tissues. The 2.8 kb transcript is found primarily in proximal regions of the intestine (D, J) where as the majority of the 2.0 kb transcript is found in mid and distal regions (J, I). It has been speculated that the multiple transcripts coding for a particular protein are due to differences in the 3'-untranslated regions, a consequence of tissue-specific alternative polyadenylation (12). It is not clear though if the difference would affect the characteristics of the final gene product. In contrast to published GLUT5 characterization studies in the rat (8,12), we did not detect the 5.1 kb transcript using in vitro transcribed antisense RNA probes complementary to GLUT5 mRNA either by decreasing the stringency of hybridization or by increasing the exposure time of autoradiography.

Solberg and Diamond (1987) compared different dietary sugars as inducers for the fructose transport. The study revealed that multiple hexoses (glc, gal, fru) may stimulate transport but fructose is by far the best inducer and likely, is the signal for the fructose transporter (18). Later studies by Buddington et al (2) showed fructose uptake was higher in mink fed the HC diet which demonstrates the ability of dietary glucose to stimulate intestinal fructose transporter. Observations from my studies provide evidence that dietary glucose does indeed stimulate GLUT5 expression at the level of transcription. The GLUT5 mRNA was up-regulated 44%, 30%, 800% within 24 h in the duodenum, jejunum and ileum respectively in response to high levels of dietary glucose. The up-regulation of the gene can be explained by two possibilities. First, dietary glucose may serve as a signal substrate for fructose transport by stimulating GLUT5 mRNA levels and therefore altering the transport capacity for fructose by increasing the number of carrier proteins. Alternatively, the transcription of the gene encoding GLUT5 is up-regulated but the translation of the gene product is not, since the concentration of dietary fructose in HC may be too low to require the increased transport capacity. Hence, the up-regulation of mRNA would essentially translate into loss of biosynthetic energy. The second possibility might be that GLUT5 transports glucose in response to changes in dietary composition, ie. when the intestine is overloaded with a high concentration of dietary glucose, in order to increase absorptive capacity and take advantage of the high energy substrate that

is available. The marked increase of 800% GLUT5 ileal mRNA might reflect the adaptive mechanism which has been previously demonstrated in the distal regions in response to an increased luminal concentration gradient of glucose (7). It is therefore conceivable that the fructose transporter may participate in the transport of dietary glucose in the course of long term adaptation to HC diets. However, further studies are required to determine whether the level of GLUT5 carrier protein is up-regulated correspondingly to its mRNA. It is interesting to note that the pattern of up/down-regulation of GLUT5 mRNA in the jejunum is similar to that of GLUT2 mRNA in response to dietary glucose (see chapter 2).

It is still not clear whether GLUT5 possesses the capability to transport glucose since published studies were carried out under different experimental conditions. Kayano et al (1990) showed expressed human GLUT5 in Xenopus laevis oocytes and found a significant 3~4 fold increase in 2-deoxy-D-glucose uptake, (a non-metabolizable glucose analogue), exhibiting sensitivity to 10 μM of cytochalasin-B. In contrast, Burant et al 1992 expressed human GLUT5 in Xenopus laevis oocytes and showed little transport of 2-deoxy-D-glc and fructose uptake was not inhibited by 100 μM cytochalasin-B. It is possible that cytochalasin-b still binds but does not exert the similar inhibitory effect on GLUT5 as with other 'true' facilitative glucose transporters since the carrier only shares 40% homology in its primary amino acid sequence with other GLUT isoforms.

Figure III.1 Immunolocalization of fructose transporter GLUT5 in rat small intestine. The panel shows the crypt and villus of a six week old adult rat jejunum. Tissue samples were incubated with rabbit anti-GLUT5 polyclonal antibodies followed by fluoresceinated goat anti-rabbit immunoglobulin G, as described in materials and methods sec. III.2. Note staining pattern was confined to the brush-border of the villus with no apparent staining in the crypt cells.

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Figure III.2 Expression of GLUT5 along the crypt-villus axis. Tissue samples collected from (A) jejunum (B) ileum show predominant staining in the brush-border membrane of the top third of the villus with decreased gradient of staining down the crypt-villus axis. The tissue sections were incubated with polyclonal human GLUT5 antibody followed by FITC-conjugated goat anti-rabbit IgG, as described in materials and methods section III.2.

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Figure III.3 Immunolocalization of GLUT5 along the horizontal axis of the rat small intestine. Tissue samples (10 μm) collected from (B) duodenum (C) jejunum (D) ileum (E) colon were incubated with rabbit polyclonal antibodies raised against human GLUT5 c-terminus peptide, followed by fluoresceinated goat anti-rabbit immunoglobulin G, as described in materials and methods section III.2. Panel (A) shows a jejunal control section subjected to 5% normal rat serum in place of the primary anti-GLUT5 Ab. Note decreased staining intensity along the small intestine horizontal axis was detected with no stainingin the colon.

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Figure III.4 Regional distribution of GLUT5 poly (A^+) RNA in rat small intestine. Poly(A^+) RNA (5 μg) isolated from duodenum (lane 1), jejunum (lane 2), ileum (lane 3), were hybridised with a 1.8 kb radiolabelled antisense [^{32}P]-CTP RNA probe, generated from BAMH1 linearized human GLUT5 cDNA in pGEM3 plasmid, which was subcloned from GLUT5;ph-JHT5. The Northern blots were performed as described in materials and methods section III.2 and the autoradiogram shown was exposed for 24h at -70°C with intensifying screen.

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Figure III.5 Regional expression of GLUT5 mRNA in response to low/high carbohydrate dietary treatment in rat small intestine and colon. A Northern blotting of total RNA (30 μg per lane; panel A) demonstrates the effects of 7 days LC feeding, followed by 1,3,7 days of HC feeding and finally, by 7 day of LC diet, from left to right, in duodenum (lane 1-5); jejunum (lane 6-10); ileum (lane 11 -14); colon (lane 16-19).(data of final 7 day LC diet for ileum and colon are not available). Northern blots were hybridized with [^{32}P]-CTP labelled antisense RNA probes, which were in vitro transcribed from BAMH1 linearized human GLUT5 cDNA in pGEM-3 plasmid. The Northerns were performed as described in the materials and methods (section III.2). The corresponding panel B shows the same Northern blots reprobed with human gamma-actin.

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Table III.1 The intestinal mRNA levels (absorbance, arbitrary units) for gamma-actin were normalised on each film to the gamma-actin level for 1 week of LC feeding in the same tissue. Thus each film gave a set of density for each lane which were normalized against the first sample of that particular tissue. The four groups of tissue examined in response to LC/HC diet were duodenum, jejunum, ileum, colon and the normalised HC treated GLUT5 RNA levels were then compared to the RNA levels treated with LC diet.

Table III.1 Summary of GLUT5 mRNA levels in high/low carbohydrate diet experiment.

Tissue/Dietary treatment	GLUT5 mRNA	Gamma-actin mRNA	GLUT5 mRNA normalized
duodenum 7LC	1.81	0.887	1.81
duodenum 1HC	3.53	1.200	2.61
duodenum 3HC	2.49	0.555	3.98
duodenum 7HC	1.0	0.194	4.57
duodenum 7LC	1.33	0.128	9.18
jejunum 7LC	8.36	5.504	8.36
jejunum 1HC	9.41	4.895	10.58
jejunum 3HC	3.96	3.144	6.92
jejunum 7HC	2.06	2.906	3.91
jejunum 7LC	4.75	4.054	6.45
ileum 7LC	0.11	3.01	0.11
ileum 1HC	0.22	0.702	0.92
ileum 3HC	1.29	3.976	0.98
ilium 7HC	0.72	3.828	0.57

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DISCUSSION

Mammalian intestinal facilitative glucose transport represents a dynamic system capable of changes in response to a variety of altered metabolic states including dietary adaptation, lactation, pregnancy, and diseases such as diabetes (19). When the carbohydrate content in the animal's diet is modulated, the intestinal epithelium can change its absorptive capacity to meet the increasing or decreasing load (11,13,20). This ability of the intestine to adapt to changing diets allows the animal to survive in times of availability of foods as the seasons change, or adapt to different vegetation as the habitat changes or the animal moves on. The objective of this thesis was to investigate the underlying molecular mechanisms controlling this response and to understand how the expression of intestinal facilitative glucose transport is modulated in the course of dietary adaptation.

The following section of the discussion is devoted to the experimental controls which were carried out with the various techniques used. Other control alternatives are also discussed in the context of appropriateness and sensitivity.

Controls for northern blotting

Quantitative comparison of mRNA levels poses some challenges since absolute amount of total RNA in each sample is required before an accurate comparison between lanes of RNA can be made. It is not always possible to load precisely the same amount of RNA, hence we ensured normalization reprobing Northern blots with human gamma-actin as an internal control. The actin genes have been used in many hybridization studies in the past. However, the issue of biological variability of these constitutively expressed house keeping genes persists. Recent studies have demonstrated usage of 28S ribosomal RNA levels, instead of actin, as a good indicator for relative amounts of RNA loaded (10). It appears that the level of 28S ribosomal RNA proved to be a constant fraction of total sample in comparison to other internal controls previously used in hybridization studies. Measurement of 28S rRNA bands is easily carried out, and avoids an extra step of rehybridization. A

photographic negative of the 28S and 18S stained gel can be scanned and the density of the bands used to normalize comparisons between lanes on the gel. In addition, the integrity of sample RNA can be assessed by the ratio of 28S/18S rRNA levels, which should be close to 2.0. A lower ratio would reflect partially degraded RNA which is primarily due to loss of the 28S rRNA band. This is anticipated because of the greater susceptibility of the larger RNA species to degradation (8,10).

Controls for western blotting

Western blot analyses were employed to quantitate levels of GLUT2 carrier protein expression occurring in the BLM. For such an analysis to be truly quantitative, there are two factors of great importance which must be considered. 1) consistency of enrichment between different preparations of purified BLM vesicles, and 2) the absolute amount of total protein in each sample. It is essential that the degree of enrichment is the same for each BLM preparation because contamination with connective and endothelial tissue would easily alter the concentration of BLM isolated. A final assessment of purified BLM can be achieved by assaying for ouabain-sensitive Na^+/K^+-activated ATPase activity, a basolateral membrane marker. This reveals the degree of BLM enrichment with respect to the initial homogenate. However, this assay should be carried out initially for each type of preparation in order to ensure consistency in the isolation procedure. Ten to eighteen fold enrichment of isolated BLM has been reported depending upon the procedure used (12,23). A less tedious method of assessment would be to obtain antibodies raised against the classical basolateral membrane marker, the RLA class 1 histocompatibility complex (17,22). The antibody can then be used directly in the western blot procedure. Dyer and colleagues have reported that an antibody against RLA class 1 antigen, reacted strongly (enterocyte basolateral membrane protein) at 43 kDa in rabbit (12). Our laboratory has attempted this procedure by using antibody against rat RLA class 1 antigen (OX18) but further optimization is required.

To determine the total protein concentration, we used spectrophotometric determination at absorbance wavelength 280, using BSA as standard. Subsequently,

the proteins are subjected to SDS-PAGE and electrolytic transfer. Staining procedure were then carried out to ensure the proteins were homogeneously bound to the nitrocellulose matrix. Ponsceau S was the protein stain of our choice, however, a more sensitive staining procedure using amido black or coomassie blue R-250 are also possibilities but more difficult to destain (3).

Controls for immunocytochemistry

A variety of problems due to background and nonspecific labelling in immunocytochemistry can be detected by performing controls with experimental tissues. There are four controls which I have carried out for all GLUT5 immunolocalization experiments:

1) incubation of intestinal tissues in PBS alone to determine if autofluorescense was occurring in some cell types. It appears the mast cells, situated in the lamina propria, possess certain autofluorescense properties because these cells are consistently brighter than all other cell phenotypes before any primary or secondary antibody treatment.

2) perform immunocytochemistry without the primary anti-GLUT5 polyclonal antibody (BBG5). This tests whether the FITC-bound secondary antibody is contributing to a high background (non-specific fluorescent staining).

3) subject the intestinal tissue to 1-5% normal rat serum (pre-immune serum) to test for non-specific binding.

4) Rat colon was used as a negative control tissue since the region does not express the GLUT5 protein carrier.

At the time when this project was initiated, information on GLUT5 localization to various tissues was not known, hence, a positive control tissue was not available to us for comparisons. Another control which proved to be quite useful in determining the specificity of the antibody was to block specific binding of the primary antibody by adding purified GLUT5 antigen (7). Incubation of the tissue with the antibody preabsorbed with the purified antigen should show a lack of immuno-staining.

The fixative used successfully in preparing intestinal tissue for immunocytochemistry was developed by I. Mclean and P.K. Nakane (24). The fixative contained 2% paraformaldehyde, 0.01 M periodate, 0.075 M lysine in 0.037 M phosphate buffer (PLP). Unlike conventional fixatives (paraformaldehyde, glutaraldehyde) which target primarily proteins and lipids, PLP is directed toward the carbohydrate as well as protein and lipid moieties. The periodate in the fixative is used to oxidize the carbohydrate moieties in the intestinal tissue to form free aldehyde groups. Lysine, a divalent amine, then cross-links the carbohydrate containing molecules by reacting with these aldehyde groups. In addition, the concentration of paraformaldehyde is high enough to achieve some stabilization of proteins and lipids but low enough so that the antigenicity is preserved.

Intestinal GLUT2

Cloning and characterization of liver GLUT2 has led to the subsequent identification and localization of the same carrier in the intestine, kidney and the pancreatic B-cells (27,29-31). Based on the comparison between restriction enzyme mapping and partial sequencing data of GLUT2 cDNA clones isolated from these different tissues, it is concluded that all GLUT2 isoforms possess similiar characteristics and perform similar functions. Although the size of the mRNA transcript is identical in the GLUT2 isoforms, Thorens et al. reported that the proteins expressed have different apparent molecular masses including 61 kDa in intestine, 53 kDa in liver and 57 kDa in kidney. They suggested the difference was due to post-translational modifications but were not able to deduce the underlying reason. It was speculated that the difference in molecular weight may be accounted for by differences in the single N-linked oligosaccharide side chain or was caused by additional modifications such as phosphorylation or proteolysis, but so far the differences have not been explained.

The identity between the intestinal and liver GLUT2 remains unclear since the total intestinal GLUT2 cDNA sequence has not been elucidated. The only sequence information known for intestinal GLUT2 is the partial sequence towards the 5' and

3' end of the cDNA which indicated base substitution at positions 1509 and 1528, the latter resulted in changing threonine to an alanyl residue. The intestinal GLUT2 also uses an alternative polyadenylation site the significance of which is not known (26). In addition, the restriction enzyme mapping suggested similar profiles between intestinal and liver cDNA, however, this information is insufficient to detect any base substitution or small insertions and deletions which may occur between the restriction sites.

Previous data from our laboratory does not entirely support the view that intestinal GLUT2 possesses similar characteristic as liver GLUT2. The attempt to localize GLUT2 within the intestine by using antibodies raised against liver GLUT2 proved to be unsuccessful. However, the monoclonal antibody recognized the positive control tissues, rat liver and the B-cells of the rat pancreas, which suggests the epitope that GLUT2 monoclonal antibody recognizes does not exist or is modified within the intestine. Furthermore, Cheeseman et al (6) clearly demonstrated that the affinity constant for cytochalasin-B binding is quite different in the enterocyte basolateral membrane compared to that reported for the liver membrane (2). It is therefore conceivable that the differences between liver and intestinal GLUT2 may lie in the functional characteristics of the protein in two entirely different tissues.

Intestinal GLUT5

At the time the dietary study was undertaken the design of the project was based on the hypothesis that GLUT5 transported glucose. Hence the rationale was to measure the level of glucose transporter mRNA in response to its changing substrate, dietary glucose. It was not until recently that the function of GLUT5 has been shown to be the transport of predominantly fructose. This finding changed the focus of the GLUT5 project and complicated the interpretation of subsequent results. The stimulatory effect of dietary glucose on fructose transporter mRNA, GLUT5, is not as high as for the glucose transporter mRNA, GLUT2. After 1 day of HC feeding, GLUT5 mRNA merely up-regulated 44%, 30% (compared to GLUT2

mRNA, 314% and 212%) in the duodenal and jejunal regions respectively. It was to be expected that glucose would stimulate the induction of its own carrier mRNA. However, the slight elevation in GLUT5 mRNA suggests two possibilities, 1) that dietary glucose enhances fructose transport by stimulating its mRNA, or 2) GLUT5 carrier protein also functions as a glucose transporter. Further investigation is required to measure the level of GLUT5 carrier protein so that it can be correlated with the GLUT5 mRNA level. The GLUT5 polyclonal antibody (BBG5) our laboratory has raised against human GLUT5 C-terminus peptide does not recognize the denatured blotted rat GLUT5 protein in western blotting analysis, but does function well in immunocytochemistry with intact rat intestinal tissue.

Another interesting finding with the GLUT5 Northern blots was the elucidation of two mature mRNA transcripts encoding for GLUT5. The transcripts not only exhibited regional specificity but also showed a considerable size difference of 800 base pairs which would most likely lead to two different sizes of translated protein. Whether the differences reflect on functional activity would require further investigation. Davidson et al (9) speculated the size difference was due to alternative polyadenylation sites but so far there has been no supporting evidence for the hypothesis. Another possibility which has not been considered is alternative splicing. This is a process whereby the introns and exons of the primary transcript are processed to form two or more alternative spliced RNA products which are subsequently translated into distinct proteins. The cell regulates the splicing process by determining the splice site of mRNA to be produced. Furthermore, the exon changes caused by alternative splicing do not produce radically different proteins, instead, they produce a set of proteins, comparable in function, modified to suit the particular needs of the tissue. Ultimately, sequence data of both RNA transcripts is still required to localize where the apparent size difference lies. This can be easily achieved by gel purifying the two mRNA transcripts, reverse transcribing them; designing several PCR primer combinations based on rat GLUT5 sequence targeting the region of interest, amplifying the target sequences and looking for heterogeneity in size between the PCR products amplified from both RNA transcripts, performing

restriction mapping analysis and comparing profiles and finally, sequence the PCR products directly by using automated sequencer (Applied Biosystems), without resorting to conventional cloning and sequencing methods which can be long and tedious.

Dietary treatment experiments

The significance of the pulse increase in jejunal mRNA levels after only 24 h of switching from low to high carbohydrate diet is unclear. The increased mRNA need not contribute to the modulation of BLM carrier expression as the rate of translation may not have changed. A second possibility remains in that the initial increase in the BLM carrier in response to HC could very well result from the up-regulation of mRNA, in which case the event may trigger a sequence of mechanisms which eventually leads to a greater up-regulation of GLUT2 carrier protein without a further increase in mRNA. In fact, the following down-regulation of GLUT2 mRNA to the level of control may serve as a fine-tuning mechanism in protecting the proximal regions of the intestine from overloading with carbohydrate. Conversely, the ileal mRNA showed a different pattern of response by not down-regulating its mRNA after the 24 h HC feeding. It is conceivable that the ileal epithelium may increase its capacity to absorb the spillover hexoses from the jejunum. However, further investigation is required to measure ileal GLUT2 proteins levels in relation to its respective mRNA.

Post-transcriptional control of dietary adaptation may be viewed from an bioenergetics perspective whereby increasing translational efficiency or up-regulating post-translational mechanisms may be more energetically favourable compared to increasing the transcription of GLUT2 mRNA, if it is not necessary.

It is unlikely that post-transcriptional mechanisms are solely responsible for the adaptive changes observed within the small intestine in response to high glucose diets. Our observations were limited to only one high carbohydrate test diet. It is conceivable that an increase from 31-59% in glucose content may not be enough to require a sustained increase in transcription within the jejunum. Therefore, a range

of glucose concentration test diets should be tried as well as the time course of diet should be extended to further explore the dynamics of cellular control mechanisms.

It should be noted that the regular daily rat chow consists of 55% carbohydrate in content, a mere 4% lower than the high carbohydrate test diet. Hence, the intestine may not need to expend the extra energy to make more message after the initial pulse increase in mRNA.

Recent developments in adaptive dietary studies have proved differently the mechanisms which the intestine utilizes to capacitate the increase in dietary sugar (glucose and fructose). The test diets given to the rats in the following two cases discussed were quite different from those used in our experiments. Miyamoto and colleagues investigated the effects of dietary sugars and found a high glucose diet stimulated the transport activity and increased the levels of SGLT1 and GLUT2 mRNA in rat jejunum. A similar response was induced for jejunal GLUT2 in response to high D-galactose and D-fructose diets. However, rat jejunal GLUT5 mRNA levels was increased significantly only in response to high D-fructose diet. The rats were given a carbohydrate-free diet for 2 weeks then switched to a 55% carbohydrate diet for 5 days (0-55% sugar). After a 3 day change from 0-55% HC diet, the GLUT2 mRNA increased 2.1 fold and it was demonstrated that the transcriptional rate increased to up-regulate GLUT2 mRNA since the rate for GLUT2 mRNA synthesis was significantly increased by day 5 of HC feedings. Conversely, the transcriptional rate and mRNA level of GLUT5 did not change after switching to the high glucose diet suggesting that GLUT5 mRNA does not respond to the change in glucose concentration and therefore does not transport glucose (26). This result is in contrast to that obtained by E. Rand and workers, which demonstrated the ability of rat GLUT5 to transport glucose in a Xenopus oocyte system (28).

Inukai and co-workers have shown that GLUT5 protein and mRNA are markedly increased in the jejuna of rats fed a fructose-enriched diet. The control rats were maintained on a 52% carbohydrate in content standard rat chow for 3 and 15 days and the test rats were given a fructose-enriched diet composed of 50% (W/W)

fructose and 26% of other carbohydrate (52-76% increase). Rat GLUT5 mRNA and protein were increased 2.5 and 6 fold respectively for 3 days of high fructose diet in comparison to the control group. Similarly, GLUT5 mRNA and protein increased 5 and 8 fold, respectively, after 15 days of fructose feeding. From this study, it was suggested that transcriptional mechanisms play an important role in the adaptive changes of the jejunum in response to increased fructose uptake (19).

Future directions

Proposal #1

Antisense technology using complementary RNA probes can be further extended by performing in situ hybridization to investigate in which subpopulation of enterocytes is the glucose carrier induced. In situ hybridization techniques rely upon the hybridization of a specifically labelled nucleic acid probe to the cellular RNA in individual cells or tissue sections. Hence, isolating the subpopulation of enterocytes responsible for the pulse increase in mRNA after 24 h of HC feeding would clearly indicate to us where the signal is perceived. For instance, where along the axis does the diet-dependent difference in glucose carrier site density take effect? Is it in the mature enterocytes which already express the carrier? Is it along the lower villus axis where the immature and undifferentiated enterocytes are localized? Or is it in the crypt stem cells? This would provide evidence as to whether the increased sugar transport depends on inducing more carriers in existing cells or producing new cells with a higher density of carriers.

Pilot studies by Ferraris and co-workers have shown an increase or decrease in phlorizin binding site density (SGLT1) appearing first in the crypt and then marching over the course of several days to the villus tip in response to increasing/decreasing glucose diet (14,15). In situ hybridization can be carried out for SGLT1, GLUT2, and GLUT5 to determine first the pattern of message distribution along the different cell populations on the crypt-villus axis and second, the level of its respective mRNA in the crypt and along the axis in response to change in dietary glucose (providing transcription is the primary mechanism involved in responding to

dietary change).

The pattern of mRNA expression has already been established for both GLUT5 in rat and SGLT1 in rabbit along the crypt-villus axis. In situ hybridization studies revealed highest levels of GLUT5 expression being at the lower to midvillus region with weaker signals extending toward the crypt-villus junction and the villus tip (28). As for SGLT1, the mRNA was detected in all regions of the villus with increasing levels from the base to the tip of the villus (18). The difference has been attributed to possibly two programs governing the expression of genes expressed in the small intestine.

Proposal #2

Cloning of rat GLUT5 was initiated by using degenerate primers based on human GLUT5 cDNA sequence to amplify the intestinal cDNA generated from reverse transcription of rat intestinal polyadenylated RNA (25). The rabbit GLUT5 polyclonal antibody raised against human GLUT5 c-terminus, which recognizes the intact rat GLUT5 protein in immunocytochemistry, does not bind to the denatured form of the protein in western blot analysis. This lead us to believe that partial inter-specie variation lies within the c-terminus domain of GLUT5. Recent sequence data of rat GLUT5 published by E. Rand et al, 1993, proved that the variations indeed occurred within the c-terminus:

human:	KEL:::PPVTSEQ
rat:	KELNDLPPATREQ

Northern blot analysis of intestinal GLUT5 showed two mature RNA transcripts encoding the protein. There is an apparent size difference of ~800 kb between the two transcripts and both exhibited regional specificity along the small intestine. It would be of considerable interest to localize the sequence differences and perform subsequent functional studies using xenopus oocyte assays to probe for differences in the biochemical properties of the fructose transporter. The functional characterization of the multiple transcripts encoding GLUT5 may resolve the

contradicting reports of whether GLUT5 is truly capable of transporting glucose in rat.

Proposal #3

The third proposal suggests the investigation and possible cloning of a facilitative glucose transporter in the brush-border membrane of the small intestine. Several reports by Brot-Laroche and colleagues have demonstrated kinetically the existence of two distinct D-glucose transport systems in the BBM vesicles of guinea pig jejunum (4). The first system identified as SGLT1, is the classical D-glucose/Na^+ cotransporter, fully inhibitable by D-glucose, D-galactose and alpha-methylglucoside. System two (SII) characteristics resemble those of GLUT2, the basolateral facilitative glucose/fructose carrier by showing substrate specificity for D-glucose, D-galactose, D-fructose but SII was not inhibited by alpha-methylglucoside, which is a potent competitive inhibitor for SGLT1 (5). It is also demonstrated that SII is sensitive to cytochalasin-B inhibition, a competitive inhibitor of facilitative glucose transporters (4). It appears that D-glucose transport in guinea pig vesicles is very temperature-sensitive and causes kinetic differences in the nature of D-glucose transport as the temperature is modulated. In the presence of Na^+ at 35 °C, two saturable systems are detected (SI with Km=0.4 and SII with Km=24 mM), with SII being saturable at a glucose concentration much higher than that needed to saturate SI. However, at 25 °C, only SI showed saturation (with Km=1.2 mM) while the SII system exhibited diffusion like kinetics (4). This indicates that SII can capacitate a wide range of glucose concentrations in the lumen without being saturated. The physiological implications of such a system would be most appropriate during the course of a meal (glucose=50 mM)(16), where absorption of the bulk of the sugar cannot take place through SI; SGLT1, which is largely saturated and has a small capacity. It is suggested that the two systems work in concert where SII takes care of the initial, rapid processing of the sugar load after a meal and SI would ensure that no free sugar remains in the lumen during periods of fast (1).

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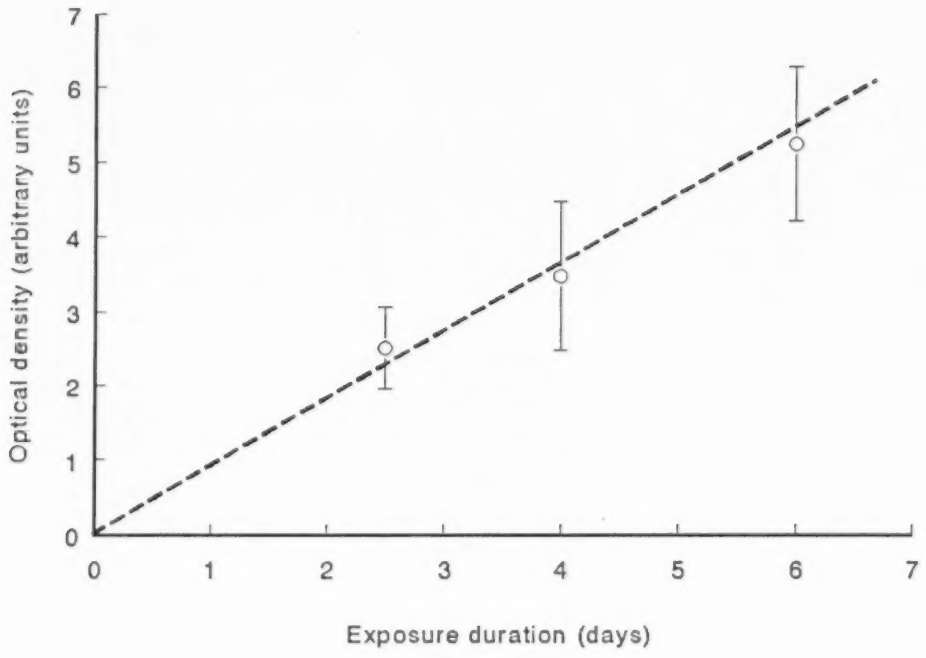

Figure A.1 Standardization curve for autoradiograms from Northern blots exposed for different periods of time. Each point represents the optical density of the 2.8 kb band detected for GLUT2 mRNA on a Northern blot. The same autoradiograms were exposed for 2.5, 4 and 6 days before development and were scanned and the values averaged. Error bars represent standard error of the mean. There is a clear linear relationship between exposure time and optical density.